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The Effects Of Courtship And Pairing Behavior On The Nonapeptide And Noradrenergic Systems Of Adult Male And Female Zebra Finches

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THE EFFECTS OF COURTSHIP AND PAIRING BEHAVIOR ON THE NONAPEPTIDE AND NORADRENERGIC SYSTEMS OF ADULT MALE AND FEMALE ZEBRA FINCHES

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DEDICATION

This work is dedicated to my wonderful parents, Pat and Cheryl Lowrey, for instilling in me the values of education, motivation, and hard work. Thank you for your unconditional love, belief, and support. To my brother, Patrick Lowrey, for his love, support, and constant cheerleading. To Dr. Tina Lowrey, Dr. L.J. Shrum, and Dr. Ronald Hess for setting the bar so high. Your love, support, and advice are greatly appreciated and you continue to inspire me. I have been blessed to have so many wonderful friends and loved ones in my life and for this, I shall always be grateful. Finally, to my incredible husband, Michael John Ondercin, who met me in graduate school and still wanted to marry me. I cannot imagine reaching this goal without you and I will always be grateful for your love and support during this long and arduous journey. I love you more than I can ever say.

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CHAPTER 1 - BACKGROUND LITERATURE

Social relationships are complex and therefore likely require the coordination of multiple neurochemicals active within neural circuits for their formation and maintenance. These circuits may include pathways regulating learning, memory, motivation, reward, and attention. Two neurochemical systems have been implicated in social relationships: catecholamines (norepinephrine and dopamine) and nonapeptides (oxytocin and vasopressin). The avian homologue of oxytocin (OT) is mesotocin (MT) and the avian form of vasopressin (AVP) is vasotocin (VT).

The Zebra Finch as an Animal Model for Social Relationships

There are several benefits to using zebra finches as a model for social relationships. First, it is important to establish the neural correlates of monogamous pairing in more than one animal species. A popular animal model of monogamy has been the prairie vole. Zebra finches use visual and vocal cues, similar to humans, while prairie voles and other rodents rely mostly on olfactory cues. Zebra finches live in large social groups, whereas prairie voles are aggressive toward others outside the family unit. Zebra finches are an excellent model of learning and memory, the neurobiology of the song system is well understood, and the genome is fully sequenced and well-annotated. In mixed-sex group-housed animals, fewer than half form a pair relationship within 24 hr, but over 75% of animals pair in 48hr (unpublished data from our lab). In contrast, prairie voles will pair after 24 hours of cohabitation with a single, opposite-sex conspecific, indicating that zebra finches are more selective than are prairie voles in their pairing.

For these reasons, zebra finch social relationships appear to have more in common with human relationships than do prairie vole relationships.

Communication is a vital component to the formation of social relationships in the zebra finch (Zann, 1996). During development, it is important for both males and females to learn their tutor's song. Males will use this song to develop their own unique song and females will use it as a template to evaluate male song in the future (Konishi and Akutagawa 1985; Marler 1991; Riebel 2000; Funabiki and Konishi 2003; Riebel 2003). Zebra finches recognize and distinguish their parents and pair bond partners from familiar conspecifics by their songs and calls (Zann, 1996). The brain areas involved in song production and audition are analogous to those in the human auditory system (Bolhuis, Okanova, & Scharff, 2010; Doupe & Kuhl, 1999; Jarvis, 2007; Moorman et al., 2012), making zebra finches an excellent model for human language acquisition and communication.

The Song System

The avian song system is well understood (Nottebohm, Alvarez-Buylla et al. 1990; Nottebohm 1991; Nottebohm 2002; Reiner, Perkel et al. 2004), and is an excellent experimental model for vocal communication, learning, and memory. Male zebra finches develop a unique song based on song learned from their fathers, or "tutors," during a sensitive period and other males in their colony (Konishi and Akutagawa 1985; Marler 1991; Funabiki and Konishi 2003). Furthermore, the brain areas underlying the song system have been implicated in learning and memory (Bolhuis, Zijlstra et al. 2000; Bolhuis, Hetebrij et al. 2001; Terpstra, Bolhuis et al. 2004; Bolhuis and Gahr 2006).

The song system contains two major pathways: the sensorimotor pathway and the anterior forebrain pathway (AFP; (Solis, Brainard et al. 2000); see Table 1). The sensorimotor pathway is necessary for normal song production throughout life and consists of the HVC (proper name), robust nucleus of the arcopallium (RA), and the tracheosyringeal portion of the hypoglossal nucleus (nXIIts; (Solis, Brainard et al. 2000). RA also projects to nuclei involved in the control of respiration. The AFP is a basal ganglia-forebrain circuit important in evaluating song feedback and modifying vocal output (Solis, Brainard et al. 2000). The AFP includes Area X, the medial nucleus of the dorsolateral thalamus (DLM), and lateral magnocellular nucleus of the anterior nidopallium (LMAN; Solis, Brainard et al. 2000). Neurons in the song system nuclei HVC, LMAN, Area X and RA are activated when the bird is singing (Solis, Brainard et al. 2000; Bolhuis and Gahr 2006). Female zebra finches do not produce song, but have a song system similar to males, although with significantly smaller (or non-existent, as is the case with Area X) nuclei (MacDougall-Shackleton, Hulse et al. 1998).

Song is an important method of vocal communication in song bird species and one of the primary functions of male song is thought to be female attraction (Searcy & Yasukawa, 1996). Female zebra finches showed a preference for an individual male's directed song, and this effect is strongest for the directed song of the female's mate (Woolley & Doupe, 2008). Although male directed song is extremely important in courtship and initial pair bond formation (Tomaszycki & Adkins-Regan, 2005), it is not necessary to maintain a formed pairbond as demonstrated by the continued paired status between females and surgically muted or song altered males (Tomaszycki & Adkins-Regan 2006).

The Auditory System

Since females do not sing, but instead choose males on the basis of song (Tomaszycki and Adkins-Regan 2005), the auditory pathway is important for adult females. The auditory pathway consists of the caudal medial mesopallium (CMM), caudal medial nidopallium (NCM), and Field L (Jarvis and Nottebohm 1997). The auditory pathway does have reciprocal connections to the song system. Exposing zebra finches to conspecific song leads to increased neuronal activation in the NCM and CMM, but not in the song system (Mello, Vicario et al. 1992; Mello and Clayton 1994; Bolhuis, Zijlstra et al. 2000), indicating that these brain areas have a specialized function for song recognition. The expression of immediate early genes (IEGs) (Sagar, Sharp et al. 1988), primarily ZENK (an acronym of zif-268, egr-1, ngfi-a and krox-24; (Moorman, Mello et al. 2011), have been used as a proxy for neuronal activation.

The findings of several studies indicate that the NCM and CMM are involved in both the processing of perceptual information involving song complexity and in the storage of song memory in songbirds and parrots (Sockman, Gentner et al. 2002; Bolhuis and Eda-Fujiwara 2003; Eda-Fujiwara, Satoh et al. 2003). Both male and female finches prefer their tutors' song to novel song (Miller 1979; Riebel, Smallegange et al. 2002; Riebel 2003), which suggests that they have learned the characteristics of tutor song and have formed an auditory memory of it (Bolhuis and Gahr 2006). In males, this 'template' is incorporated into their own song (Konishi and Akutagawa 1985; Funabiki and Konishi 2003). In females, the template is used to evaluate male song quality in mate choice (Riebel 2000; Riebel 2003). During development, ZENK is directly regulated by song in the NCM during tutor song memory acquisition (Jin and Clayton 1997; Stripling, Kruse et al. 2001; Gobes, Zandbergen et al. 2010). Bilateral neurotoxic lesions of the NCM of adult male zebra finches impairs the recognition of tutor song, but not the ability to

discriminate other bird calls or the ability to produce song (Gobes, Zandbergen et al. 2010). Thus, the NCM plays a specific role in memory for tutor song and not attention-based discrimination. Electrolytic lesions of the CMM, but not lesions of the HVC, disrupted the ability of female zebra finches to discriminate conspecific from heterospecific song (MacDougall-Shackleton, Hulse et al. 1998). Taken together, these findings indicate a unique role for the NCM and CMM auditory areas in song learning and memory. By extension, these regions should play an important role in female song perception and mate choice, a vital component of pairing.

Hypothesis for Ch.2: Creating a social relationship, such as a monogamous pairbond, will increase nonapeptide expression in the paraventricular nucleus (PVN) and bed nucleus of the stria terminalis (BSTm) of both male and female zebra finches.

Nonapeptides and Monogamous Behavior

The prairie vole is the most popular mammalian model for monogamous social relationships. There is evidence to suggest that both AVP and OT are important for formation of the pair bond in male prairie voles. Monogamous male voles have higher OTR densities in the nucleus accumbens (NAc) than non-monogamous males (Ophir, Gessel et al. 2012). OT receptor (OTR—the only OT receptor class) density in the posterior portion of the insula predicts mating success in males (Ophir, Gessel et al. 2012). Chronic intracerebroventricular infusions of AVP result in selective partner preference in male prairie voles in the absence of mating, which can be reversed by administration of a V1aR antagonist (one of the primary AVP receptors expressed in the brain; Winslow, Hastings et al. 1993). These studies indicate a role for both nonapeptides in monogamous behavior in the male prairie vole.

The relationship between OT and social attachments (both pairing and maternal) has been the focus of much female prairie vole research. Extracellular concentrations of OT, measured by in vivo microdialysis, were increased in the NAc of female prairie voles during unrestricted interactions with a male, irrespective of copulation (Ross, Cole et al. 2009). Inducing over-expression of OTRs in the NAc of female prairie voles resulted in accelerated partner preference formation after 48 hr cohabitation with a male, but had no significant effect on alloparental behavior (Ross, Freeman et al. 2009). These same manipulations in the non-monogamous meadow vole had no behavioral effect (Ross, Freeman et al. 2009). The distribution of OT-immunoreactive (OT-ir) fibers is quantitatively similar across prairie voles, meadow voles, rats, and mice. However, prairie voles have significantly higher OTR distribution compared to other species (Ross, Cole et al. 2009). This indicates a strong relationship between OT and OTR expression and female social behavior in the prairie vole. However, there is also evidence to suggest that the sex difference between the nonapeptides may be more complex than previously thought. Administration of both OT and AVP and 1 hr cohabitation increased social contact with familiar partner in both male and female prairie voles (Cho, DeVries et al. 1999).

Administration of OT+OT antagonist or AVP+AVP antagonist was associated with lower levels of social contact in both sexes (Cho, DeVries et al. 1999). This indicates that both OT and AVP are important for social contact in both males and females, but only at high levels.

Despite the abundant molecular and behavioral evidence implicating the role of nonapeptides in social monogamy, these results do not always translate to other species. There is a great deal of evidence demonstrating that V1aR expression in the ventral pallidum (VP) is critical to monogamous behavior in the prairie vole (Liu, Curtis et al. 2001, Lim, Wang et al. 2004, Lim

and Young 2004, Young, Liu et al. 2008). However, male prairie voles that demonstrate promiscuous behavior in nature do not have significantly different levels of V1aR expression in the VP than do males demonstrating monogamous behavior (Ophir, Wolff et al. 2008). There are other animal species that exhibit monogamous behavior and do not have high densities of V1aR in the VP, such as Peromyscus mice (Turner, Young et al. 2010) and the zebra finch (Goodson, Evans et al. 2006). Therefore, it is possible that these receptors may play a permissive role in monogamous behavior, rather than dictating it (Ophir, Wolff et al. 2008, Goodson 2013). This may also be true for the OTRs and the role they play in monogamous behaviors. OTRs in the NAc are necessary for pair bonding in female prairie voles (Young, Lim et al. 2001, Liu and Wang 2003) and exogenous administration of OT via minipump promotes pair bonding in the absence of mating (Liu and Wang 2003). Viral vector-mediated up-regulation of OTR expression in the NAc promotes selective partner preference (Ross and Young 2009). A distinction needs to be made between mammalian social attachment and sexual fidelity, as many prairie vole litters have multiple paternities, indicating that prairie voles are not sexually monogamous (Ophir, Wolff et al. 2008).

Evidence for the role of nonapeptides in pair bonding in other monogamous species is scarce. In monogamous primate species, affiliative behaviors between members of a monogamous pair account for variations in urinary OT in both sexes of cotton-top tamarins (Snowdon, Pieper et al. 2010), but blocking OT did not alter the preference for a monogamous partner over an opposite-sex stranger in marmosets (Smith, Agmo et al. 2010). OT effects on social behavior are not exclusive to monogamous species, as the promiscuous chimpanzee has higher urinary OT after exhibiting prosocial food-sharing behavior (Wittig, Crockford et al.

2014). A monogamous fish species, the cichlid, shows no effect of nonapeptide treatments on monogamous behavior (Oldfield and Hofmann 2011). OT administration has been shown to increase many affiliative behaviors including trust and cooperation (Baumgartner, Heinrichs et al. 2008; Delgado 2008; De Dreu, Greer et al. 2010), positive communication (Ditzen, Schaer et al. 2009), empathy (Bartz, Simeon et al. 2011), generosity (Zak, Stanton et al. 2007), social recognition memory (Rimmele, Hediger et al. 2009), and interactions with a romantic partner in humans [reviewed in (Bartz and Hollander 2006; Feldman 2012; Guastella and MacLeod 2012; Yamasue, Yee et al. 2012)]. However, a major criticism of these findings is that other explanations for OT's effects on behavior, such as general anxiety reduction, have not been ruled out (Churchland and Winkielman 2012). Also, the negative findings related to OT activation have not been explained, such as maternal aggression (Bosch, Meddle et al. 2005), paternal care (Wittfoth-Schardt, Grunding et al. 2012), feelings of envy and gloating (Shamay-Tsoory, Fischer et al. 2009), ethnocentrism (De Dreu, Greer et al. 2011), and out-group derogation (De Dreu, Greer et al. 2010). Thus, the role of OT in social relationships across species is not well understood.

The social relationship literature promotes the view that nonapeptides regulate monogamy across species, but until very recently, the evidence for this has only been found in the prairie vole (Curley and Keverne 2005; Goodson and Thompson 2010). Sex differences in the role of nonapeptides in social relationships have been emphasized, but research has also contradicted this assertion. Exogenous administration of both OT and AVP at high doses has been shown to facilitate pair bonding in both sexes of prairie voles (Cho, DeVries et al. 1999). More recently, the role of nonapeptides in pairing has been extended to zebra finches. Research

suggests that mesotocin is important for pairing in both male and female zebra finches (Pedersen &Tomaszycki, 2012; Klatt & Goodson, 2013).

Nonapeptides and the Zebra Finch

Initial investigations of the effects of VT on social behavior in the zebra finch revealed a link between VT and aggressive behavior. Infusion of a VT antagonist in the (lateral septum) LS of male zebra finches significantly reduced aggressive behaviors, while infusion of VT in the same area significantly increased beak fencing, an aggressive behavior (Goodson and Adkins-Regan 1999). Neither treatment had any effect on directed (courtship) singing or on any other courtship or pair bonding behaviors (Goodson and Adkins-Regan 1999). However, FOS activity in VT-ir neurons of the medial bed nucleus of the stria terminalis (BSTm) of male zebra finches increases selectively in response to positive social stimuli, such as an opposite-sex conspecific (Goodson, Evans et al. 2006) over a positive non-social stimulus (a water bath; Goodson, Rinaldi et al., 2009). Also, males who reliably fail to court females have significantly fewer VT-ir neurons in the BSTm than reliable courters (Goodson, Rinaldi et al. 2009). Therefore, these findings suggest that VT may have differing effects on behavior depending on the brain area in which receptors are activated.

Mesotocin (MT) also affects behavior in the zebra finch. Administering an OT antagonist in the LS significantly reduces time spent with large groups and familiar social partners, but did not decrease total time spent in social contact with conspecifics (Goodson, Schrock et al. 2009). These effects were rescued by administering MT directly in the LS (but not by VT) supporting a specific effect on the VT3 (OTR) receptor (Goodson, Schrock et al. 2009). In five estrildid finch species (two territorial species, a moderately gregarious species, and two

highly gregarious species), species-typical group sizes correlate with MT receptor distributions in the LS regardless of sex (Goodson, Schrock et al. 2009). This research is consistent with literature from other species suggesting that MT plays a role in sociality (i.e. time spent in the company of conspecifics).

Social behaviors are modified by nonapeptide expression in the brain areas important for hearing song. Both the V1aR and OTR receptors are prevalent in the NCM and CMM (Leung, Abebe et al. 2011), auditory areas important for song memory and assessing song quality in both sexes.

Surprisingly, nonapeptide receptor mRNA has not been found in the 4 primary song areas, although studies, to date, have been qualitative (Leung et al. 2011). Some research supports the idea that the nonapeptides are not involved in singing. Directed song is not influenced by central infusions of MT, VT, or other nonapeptide receptor antagonists (Goodson and Adkins-Regan 1999; Goodson, Evans et al. 2004; Kabelik, Schrock et al. 2011). Furthermore, lowering VT production in the BSTm via VT antisense oligonucleotides also had no behavioral effect on directed singing in males (Kelly, Kingsbury et al. 2011). Chronic infusions of a VT antagonist do not impair natural pair bonding behaviors in males when they are in a colony environment (Kabelik, Schrock et al. 2011). However, other studies support the idea that nonapeptides are involved in singing. V1aR receptors are present in regions such as the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc), areas that project to the song system (Gale, Person et al. 2008; Leung, Abebe et al. 2011). Systemically administered OT antagonists decrease singing in a colony environment (Pedersen & Tomaszycki, 2012). Also, males who reliably fail to court females have significantly fewer VT-ir neurons in the BSTm

than reliable courters (Goodson, Rinaldi et al. 2009). These results indicate a further need to explore the role of nonapeptides in male singing.

There is evidence to suggest that nonapeptides, particularly MT, do play a role in pairing behavior in the zebra finch. Administration of an OTA results in a significant decrease in pairing behavior in both sexes (Pedersen and Tomaszycki 2012). Furthermore, OTA treated birds that do pair are less likely to remain paired, independent of sex (Klatt and Goodson 2013). Collectively, recent research has emphasized a role for nonapeptides in pair bonding in both sexes of the zebra finch. The proposed study seeks to establish a relationship between nonapeptide mRNA density in the (paraventricular nucleus) PVN and BSTm and courtship and pairing behaviors in a semi-naturalistic experimental paradigm.

Hypothesis for Ch.3: Forming a social relationship, such as a monogamous pairbond, increases expression of catecholaminergic protein in the social behavior networks and auditory regions of both male and female zebra finches.

Dopamine (DA) has also been implicated in the formation of social relationships. DA is one of the primary regulators of monogamous pair bonding behavior in a popular animal model, the prairie vole. Research in this small rodent has shown that DA antagonists block the formation of a partner preference in mating female voles, while DA agonists facilitate formation of a partner preference in the absence of mating (Gingrich, Liu, Cascio, Wang, & Insel, 2000). Additionally, mating-induced DA release selectively activates DA receptors to promote pair bond formation in male prairie voles (Aragona & Wang, 2009). These studies indicate that DA receptors are implicated in the motivation to form a pair bond in both male and female prairie voles.

Although research across a wide array of species has demonstrated the role of DA in courtship and sexual behavior, at present, the role of DA in pairing behavior has been largely restricted to a single study species, the prairie vole. While the prairie vole has provided important insight into the neural processes of monogamous social relationships in mammals, this species relies heavily on chemosensory cues to establish a pair bond (Aragona & Wang, 2009). Thus, direct links between results from prairie vole research and less olfactory species, such as humans, primates, or birds, need to be made with caution.

There are several studies indicating that monogamous behavior activates reward pathways in the brain in the zebra finch. The ventral tegmental area (VTA) is an area of the midbrain containing a great number of DA neurons and is highly involved in reward and motivation in humans, mammals, and birds. Fibers immunoreactive (ir) for tyrosine hydroxylase (TH), which catalyzes the rate limiting step in the synthesis of catecholamines, are present in most of the auditory forebrain, thalamus, and midbrain in canaries and white-throated sparrows (Appeltants et al., 2001; Matragrano et al., 2011). Comparisons of various estrildids (finch species) that differ in their degree of monogamy and sociality have shown that the number of TH-ir neurons in the caudal VTA positively correlates with the degree of sociality (Goodson, et al., 2009). TH density in the caudal VTA was positively correlated with the production of directed song in males (Alger, Juang et al. 2011), indicating that the VTA is important for the socially rewarding aspects of directed song and courtship. To further support this line of evidence, it has been found that male zebra finches who participate in more courtship behaviors have more TH-positive cell bodies in the caudal VTA than males who do not court (Goodson, Kabelik et al. 2009). The number of songs produced by a male finch was positively correlated

with the percentage of TH-positive neurons expressing FOS in the caudal VTA (Goodson, Kabelik et al. 2009).

The vertebrate social network is made up of the brain areas: BSTm, LS, periaqueductal grey (PAG), medial preoptic nucleus (POM), and the ventromedial hypothalamus (VMH) (see Table 1 for a full list of all abbreviations; Goodson, 2005). There is an increase in the co-localization of ZENK and TH-ir in the PAG and an increase in ZENK in the VTA of males who exhibited singing behavior compared to those who were silent (Lynch, Diekamp et al. 2008). The ZENK levels in the PAG and VTA were not significantly correlated with the amount of singing, indicating that catecholaminergic neurons in the PAG are involved in the motivational or attentional components of song and not the motor output (Lynch, Diekamp et al. 2008). These data indicate that DA receptors in social reward areas of the brain are implicated in the monogamous behavior of male zebra finches.

In female white-throated sparrows, playback of conspecific male song significantly increased the phosphorylation of TH in the NCM within 15 min (Matragrano et al., 2012), suggesting that hearing song rapidly engages the catecholamine system (Maney, 2013). There is also an increase in the co-localization of TH and ZENK in the locus coeruleus (LoC), where catecholamines are synthesized, in song-exposed female zebra finches when compared to those exposed only to silence (Lynch, Diekamp et al. 2012). TH labeling density in the VTA and VMH are positively related to the amount of courtship behavior demonstrated by the partner (Alger, Juang et al. 2011). TH densities in the POM and VMH significantly explained clumping behavior (Alger, Juang et al. 2011), which is initiated by the female and is a clear sign of pair bonding (Zann 1996). Taken together, these studies demonstrate the importance of

catecholaminesin auditory and reward areas for song perception and mate choice in female zebra finches.

Hypothesis for Ch.4: Creating a social relationship, such as a monogamous pairbond, will increase activation of noradrenergic receptors in song and auditory regions of both male and female zebra finches.

Despite evidence of DA's role in social relationships, the catecholamine norepinephrine (NE) may also play a role, particularly in the zebra finch. In voles, the nonapeptide system appears to work in concert with the dopaminergic system to facilitate pairing (Aragona and Wang 2009). However, my own research has shown that these findings do not generalize to zebra finches (Lowrey & Tomaszycki, in prep.). Instead, research from our lab suggests that norepinephrine (NE) plays an important role in pair bonding in the zebra finch (Vahaba, Lacey, & Tomaszycki, 2013). Thus, zebra finches and prairie voles appear to share common nonapeptide mechanisms, but divergent catecholaminergic mechanisms. Therefore, NE may be a more likely candidate in the formation of the zebra finch pairbond than DA and this possibility should be investigated.

The physiology and function of the noradrenergic system appears to be conserved across vertebrate species (Smeets and González 2000), however, the brain of songbirds contains 10 times the amount of NE compared to mammals (Barclay and Harding 1988; Waterman and Harding 2008), suggesting that NE may play a larger role in birds than in mammals. NE receptors are present in song regions [HVC, RA, LMAN, and Area X], areas that project to the song system [VTA and the SNc], as well as auditory region (NCM), the hippocampus (Hp), the PVN, and areas that make up part of a social behavior network (Barclay, Harding et al. 1996;

Mello, Pinaud et al. 1998; Newman 1999; Riters and Ball 2002; Cornil, Castelino, & Ball 2008; Velho, Lu et al. 2012). Given the importance of NE in reward, motivation, decision making, learning, and memory as well as its prevalence in the brain of the zebra finch, NE likely plays a role in courtship and pair-bonding in both sexes (Vahaba, Lacey , & Tomaszycki, 2013).

The Noradrenergic System

NE plays an important role in the regulation of sensory responsiveness across behavioral states (Castelino and Schmidt 2010). NE cell bodies are localized to the LoC, and NE neurons show elevated tonic firing rates during vigilant, aroused, or attentive states and decreased activity during low states of vigilance (Berridge and Waterhouse 2003). NE activity in the LoC has been related to the regulation of sleep, depression, and other behavioral state dependent disorders (Castelino and Schmidt 2010). Activity in the LoC has been related to a wide array of behaviors including stress, reproduction, and aggression (Berridge and Waterhouse 2003). NE has also been associated with learning (Hu, Real et al. 2007) and memory (Davis and Squire 1984, Sarter and Markowitsch 1985). There is even recent evidence that NE works on microglia, astrocytes, and many neurons to improve overall activity of the central nervous system [see (O'Donnell, Zeppenfeld et al. 2012) for a review]. Despite the large amount of research linking NE to these behaviors, there is very little research on the role of NE in social behaviors, such as the formation of pair relationships.

Norepinephrine in the Avian Song System

The songbird auditory pathway is heavily innervated by neuromodulatory inputs. Fibers immunopositive for dopamine beta-hydroxylase (DBH), which catalyzes dopamine to NE, innervate the auditory regions in white-throated sparrows and zebra finches (Matragrano et al.,

2011; Mello et al., 1998). These DBH-ir fibers likely originate from the LoC, which clearly innervates the auditory forebrain, thalamus, and midbrain in pigeons (Kitt and Brauth, 1986). In the zebra finch, DBH-ir cells are abundant in the LoC, HP, VTA and SNc (both areas are involved in motor control, learning, and motivation) (Mello, Pinaud et al. 1998; Gale and Perkel 2006). DBH-ir fibers have also been found in the VTA and SNc innervating Area X (Castelino, Diekamp et al. 2007). DSP-4, a specific noradrenergic neurotoxin, significantly reduces the level of DBH-ir in HVC and RA and reduces both TH-ir and DBH-ir levels in Area X (Castelino and Ball 2005). Waterman and Harding (2008) demonstrated that DSP-4 treatment did not affect NE function in the hypothalamus (including PVN and POM). However, the number of DBH-ir cell bodies was decreased in treated males in the LoC and VTA (Waterman and Harding 2008). These findings indicate an important role for NE in the avian song system and associated areas.

Norepinephrine and Courtship Behavior

NE plays a role in directed song and other courtship behaviors. High rates of NE turnover have been found in Area X after exposure to a female (Barclay and Harding 1988), indicating that this area is important for motivated courtship behaviors, such as directed singing. There is evidence that NE modulates activity in brain areas involved in the social behavior network, learning, and motivation.

In the European starling, a seasonal breeder, alpha-adrenergic receptor (ADRA2) levels are higher in the POM after singing, regardless of testosterone level or season (Heimovics, Cornil et al. 2011). Collectively, this evidence demonstrates NE involvement in not only the production of song, but the motivational aspects of courtship, given its presence in areas important for learning and memory and the social behavior network.

Norepinephrine and Female Mate Choice

The NCM and CMM play an important role in song perception and mate choice in female zebra finches. ZENK is rapidly induced in the NCM and CMM in both female canaries and zebra finches in response to conspecific song versus other auditory stimuli (Mello, Vicario et al. 1992), indicating that NCM and CMM both play a role in song evaluation. There is evidence that NE is modulating this song evaluation in the NCM and CMM. Long songs, which are preferred by female zebra finches, increased levels of DBH-ir in the NCM compared to short songs (Sockman & Salvante, 2008). Studies show that blocking NE also affects female song perception and mate choice. Female zebra finches normally prefer complex conspecific male songs, but treatment with DSP-4 abolishes this preference (Vyas, Harding et al. 2008). Treatment of female canaries with DSP-4 results in reduced ZENK expression in the NCM and CMM (Lynch and Ball 2008) and a decrease sexual responsiveness to hearing conspecific male song (Appeltants, Del Negro et al. 2002). ADRA1d is the most prevalent noradrenergic receptor in the NCM and was found in more song-responsive cells in the NCM than other receptor types (Velho et al. 2012). These studies indicate that the auditory region NCM and CMM are important for female song recognition and that this behavior is mediated by NE action.

Administrations of DSP-4 have been shown to abolish the behavioral effects of NE on courtship behaviors. DSP-4 injections in male zebra finches cause a significant increase in ZENK expression in Area X associated with directed song (Castelino and Ball 2005). Administration of DSP-4 resulted in treated males taking longer to sing and performing fewer song bouts and courtship displays, but all other female-directed behaviors were unaffected (Barclay, Harding et al. 1996). There is also evidence from our lab that DSP-4 treatment has a significant effect on courtship, pairing, and female choice. Using a two choice paradigm, control females has a clear preference for control males versus DSP-4 treated males, while DSP-4 treated females displayed no preference (Vahaba, Lacey, & Tomaszycki, 2013). In an aviary setting, DSP-4 males demonstrated less directed singing than control males (Vahaba, Lacey, & Tomaszycki, 2013), which is likely the cause of the preference demonstrated by untreated females for untreated males, as they were more likely to engage in directed singing, an important courtship behavior. DSP-4 treated females displayed more clumping behavior during that first two days of testing than control females (Vahaba, Lacey, & Tomaszycki, 2013) and, combined with their lack of preference in the two choice paradigm, suggests that DSP-4 affects female choice, perhaps making females less able to assess quality mates (i.e. making them less picky). This is likely, given the evidence that DSP-4 affects female song preference (Appeltants, Del Negro et al. 2002; Vyas, Harding et al. 2008). Overall, treatments increased the latency to form a pair relationship in males, and decreased the likelihood to pair in both sexes (Vahaba, Lacey, & Tomaszycki, 2013). Combined with the molecular evidence discussed previously, this behavioral evidence strengthens the argument that NE is playing a role in not only song production, but courtship behaviors in males and mate choice in females.

Hypothesis for Ch.5: OT modulates expression of noradrenergic mRNA expression in both male and female zebra finches and effects social choice in both sexes.

I hypothesize that OT modulates the noradrenergic system by increasing the level of alpha-adrenoceptor (ADRA) mRNA. A similar effect has been reported in rodents in which subchronic treatment with OT increased the responsiveness of ADRA2 to an alpha2 agonist in the hypothalamus, amygdala, paraventricular thalamic nucleus (Diaz-Cabiale et al. 2000), and the LoC (Petersson et al., 1998). However, this hypothesis has not been tested in an accepted model of monogamous pair bonding or in birds. The nonapeptides originate in the PVN. Efferent projections extend to the LoC, LS, and VTA in avian species (Korf 1984). From the LS, VT projections extend to the BST and from there, the periaqueductal grey (PAG), indicating that VT can easily influence the social behavior network of brain regions (Goodson 2005; Goodson, Rinaldi, & Kelly, 2009). The VTA projects to several song areas, including Area X, HVC, and RA (Castelino and Ball 2005). Taken together, this evidence shows that nonapeptides have widespread influence over areas in the zebra finch brain important in courtship and pairing behaviors in both sexes.

Little is known about the mechanisms controlling the interaction between the nonapeptide and noradrenergic systems in the zebra finch brain, however, there is evidence of such a relationship in rodents (Diaz-Cabiale et al. 2000; Petersson et al., 1998). However, this hypothesis has not been tested in an accepted model of monogamous pair bonding. I seek to determine whether this same interaction between OT and NE mediates the pairing process in zebra finches.

The noradrenergic system has been well studied in the brain areas controlling song in the male zebra finch. The density of ADRA2 receptors in the song system is also higher in males than in females (Riters & Ball 2002). ADRA2 are located in Area X (Cornil, Castelino, & Ball, 2008; Riters & Ball, 2002), LMAN (Cornil, Castelino, & Ball, 2008; Riters & Ball, 2002), HVC (Cornil, Castelino, & Ball, 2008; Heimovics et al., 2011; Riters & Ball, 2002), and RA (Cornil, Castelino, & Ball, 2008; Heimovics et al., 2011; Riters & Ball, 2002). However, nonapeptide receptors are only present at extremely low levels in the song system (Leung et al. 2011).

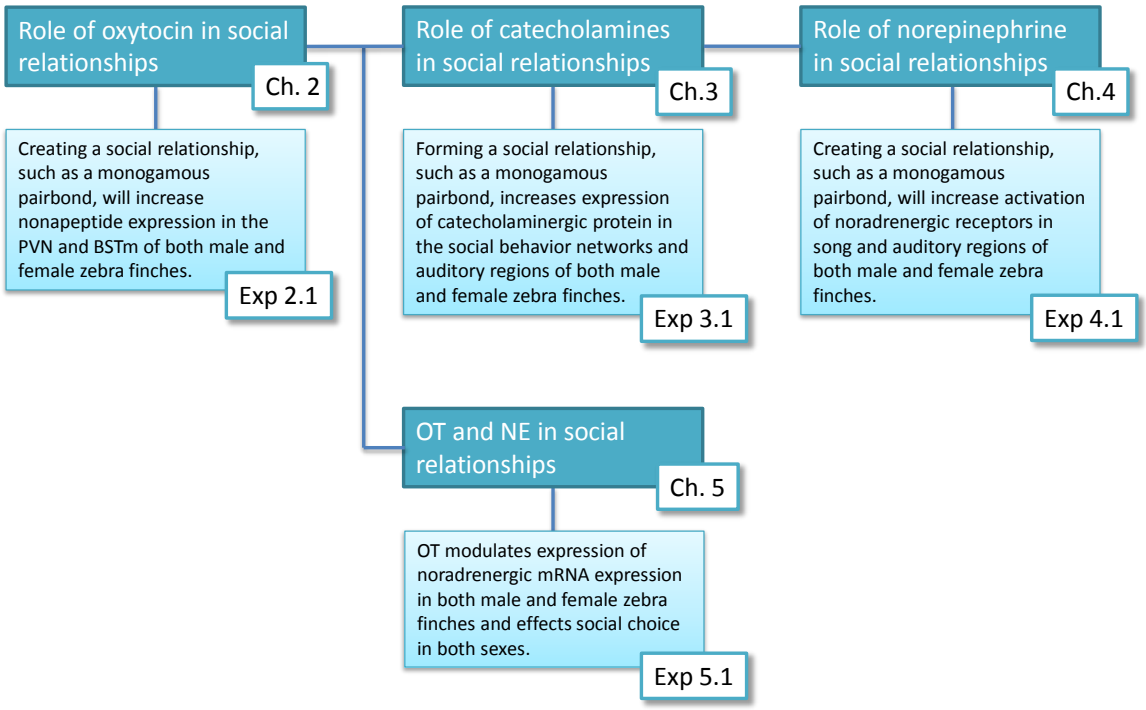
Therefore, I hypothesize that MT (originating in the PVN) is released in response to courtship behaviors, specifically directed song, and increases the level of ADRA2c mRNA in male zebra finches, which is the most prevalent receptor class within the song system (Velho et al., 2012).

In female zebra finches, who do not sing, the evaluation of male directed song is an extremely important factor in the formation of a pair bond (Riebel, 2000; 2003; Zann, 1999; Tomaszycki & Adkins-Regan, 2005). The auditory brain regions NCM and CMM are where this evaluation takes place. ADRA1d is the most prevalent receptor in the NCM and was found in more song-responsive cells in the NCM than other receptor types (Velho et al. 2012). OT receptors have also been found in the NCM and CMM (Leung et al. 2011). Therefore, I hypothesize that MT (originating in the PVN) is released in response to courtship behaviors and increases the level of ADRA1d mRNA in auditory regions of female zebra finches.

Table 1.1. A full list of brain area abbreviations in the zebra finch.

Full Name	Abbreviation
Sensorimotor Pathway	
letter-base proper name	HVC
robust nucleus of the arcopallium	RA
Anterior Forebrain Pathway (AFP)	
Area X	Area X
lateral magnocellular nucleus of the anterior nidopallium	LMAN
Auditory Areas	
caudal medial mesopallium	CMM
caudal medial nidopallium	NCM
Vertabrate Social Network	
bed nucleus of the stria terminalis	BSTm
lateral septum	LS
periaqueductal or central grey	PAG
medial preoptic nucleus	POM or POA
ventromedial hypothalamus	VMH
Catecholamine Areas	
hippocampus	HP
locus coeruleus	LoC
nucleus accumbens	Ac
sunstantia nigra pars compacta	SNc
ventral tegmental Area	VTA
Nonapeptide Production	
paraventricular nucleus of the hypothalamus	PVN

Figure 1.1. A diagram representing proposed hypotheses.



CHAPTER 2 - THE FORMATION AND MAINTENANCE OF SOCIAL RELATIONSHIPS INCREASES NONAPEPTIDE mRNA IN ZEBRA FINCHES OF BOTH SEXES

The nonapeptides oxytocin (OT) and vasopressin (AVP) are widely implicated in social behavior (Donaldson & Young, 2008; Goodson & Thompson, 2010; Young & Wang, 2004). In particular, pharmacological manipulations demonstrate that OT and AVP regulate pairing behavior in monogamous prairie voles (Cho, DeVries, Williams, & Carter, 1999; Wang & Aragona, 2004). Furthermore, monogamous prairie and pine voles can be distinguished from polygamous montane and meadow voles in nonapeptide receptor distributions (Lim & Young, 2006). Such findings in monogamous voles are often generalized to all species that form pair bonds, including humans. Such generalizations are not necessarily warranted, given the diversity of behaviors, degree of sociality, and signal modalities (olfactory vs. visual/vocal) across species that form pair bonds (Curley & Keverne, 2005; Goodson & Thompson, 2010). Indeed, findings on differences in receptor distributions between monogamous and polygamous species do not even generalize to mice (Bester-Meredith, Young, & Marler, 1999; Turner et al., 2010).

Studies on the role of nonapeptides in other monogamous species have yielded mixed results. In cotton-top tamarins, interactions between members of a monogamous pair are correlated with increased urinary OT in both sexes (Snowdon et al., 2010), but OT antagonists (OTAs) do not significantly alter partner preferences in closely related marmosets (Smith, Agmo, Birnie, & French, 2010). Similarly, there is no relationship between monogamous behavior and nonapeptides in cichlids (Oldfield & Hofmann, 2011). In humans, although OT administration has increased many affiliative behaviors, including trust, memories for socially-relevant facial

expressions, tactile contact, sex, and thinking about a romantic partner [reviewed in (Bartz & Hollander, 2006; Feldman, 2012; Guastella & MacLeod, 2012; Yamasue et al., 2012)], others have criticized these findings for not ruling out simpler functions of OT in human behavior, such as general anxiety reduction (Churchland & Winkielman, 2012). Thus, there is converging evidence for the role of nonapeptides in social relationships, but not within the context of a pair bond.

The present study examined the relationship between nonapeptides and pairing behavior in zebra finches. Studies that have used a forced-choice testing paradigm (placing one female and one male together) found no role for nonapeptides in pairing (Goodson, Lindberg, & Johnson, 2004; Kabelik, Klatt, Kingsbury, & Goodson, 2009). When using a more naturalistic paradigm that allows for a choice of partners (4 females and 4 males housed together), OTAs, administered either centrally or peripherally, decrease pair formation in both sexes (Klatt & Goodson, 2013; Pedersen & Tomaszycki, 2012). Thus, experimental evidence suggests that nonapeptides play an important role in pairing in monogamous zebra finches, but the testing paradigm is important in determining these effects of nonapeptides on pairing behavior.

Monogamy is common among birds, but the mechanisms of pairing in these species remain poorly understood. To further investigate the role of nonapeptides in avian pairing, the present study tested the effects of forming pair relationships on mesotocin and vasotocin mRNA expression (MT and VT, avian homologues of oxytocin and vasopressin, respectively) in the regions that contain these cell populations, the paraventricular nucleus of the hypothalamus (PVN, both MT and VT) and the bed nucleus of the stria terminalis (BSTm, VT) (Goodson & Thompson, 2010). Very little research has focused on the capacity for social relationships to alter

the nonapeptide system. In male prairie voles, AVP mRNA in the BSTm increases after 3 days of cohabitation (Wang, Smith, Major, & De Vries, 1994) and, in females, OT levels increase in the nucleus accumbens in response 4 hours of free access to a male, especially if mating occurs (Ross et al., 2009). Increases in nonapeptide mRNA are also evident in other behaviors: paternal and maternal behavior, as well as prolonged social isolation, increase AVP and OT mRNA in the PVN (Pan, Liu, Young, Zhang, & Wang, 2009; Wang, Liu, Young, & Insel, 2000).

We examined the number of cells expressing nonapeptide mRNA in both sexes at two time points: either 48hr or 2 weeks after pairing. We hypothesized that pairing behaviors in zebra finches would increase MT and VT mRNA in the PVN of both sexes relative to both control groups, since social manipulations result in an increase in nonapeptide mRNA in other species (Pan et al., 2009; Wang et al., 1994; Wang et al., 2000), and the PVN has diverse effects on behavior due to the simultaneous secretion of nonapeptides into the bloodstream and the brain (Goodson, 2005; Neumann, 2002). Furthermore, we chose to examine VT mRNA in the BSTm, since not only does this region secrete VT, but VT protein expression in this region is associated with male courtship behavior and positive social stimuli in zebra finches (Goodson & Kabelik, 2009; Goodson, Rinaldi, & Kelly, 2009). Furthermore, the BSTm heavily projects to the lateral septum (LS), a region implicated in a variety of socio-sexual behaviors (Goodson et al., 2009; Goodson & Thompson, 2010; Goodson & Wang, 2006; Newman, 1999). Thus, we hypothesized that pairing would also increase VT mRNA in this region, but that the effects may be more pronounced in males than in females, since AVP/VT protein expression is higher in the BSTm of males compared to females across a variety of species (De Vries & Panzica, 2006).

We had two competing hypotheses. First, we hypothesized that, if zebra finches are more like prairie voles, increases in nonapeptide mRNA would be evident in the 48hr (pair formation) group, since cohabitation with a female over this time period increases vasopressin mRNA in male prairie voles (Wang et al., 1994). Pair maintenance is rarely studied, but some research in zebra finches and in prairie voles suggests that the mechanisms may be different (Aragona et al., 2006; Tomaszycki & Adkins-Regan, 2006). Thus, for our 2 week group, we sought to determine if similar increases in nonapeptide mRNA were evident. Second, if zebra finches differ from prairie voles, then we predicted that there would be no differences in nonapeptide mRNA expression between paired and unpaired animals.

To better understand which behaviors predicted increases in nonapeptide mRNA, we used multiple linear regression analyses, similar to previous studies which have correlated behavior with protein expression of neurotransmitters or urinary concentrations of peptides (Alger, Juang, & Riters, 2011; Snowdon et al., 2010). When considering the relationship between behavior and nonapeptide expression, we based our hypotheses on our view of pairing as a 3-step process: male courtship (song), female mate choice (based on song perception, which causes the female to initiate contact), and pairing in both sexes (which is identified by contact exclusivity between two animals). Thus, we hypothesized that singing in males and pairing behaviors in females would be more likely to predict variations in nonapeptide mRNA.

Method

Subjects

Sixty-four subjects (32 animals of each sex) were adult wild-type zebra finches (*Taeniopygia guttata*) raised in social aviaries between the ages of 90 days and 3 years. Subjects

in the pairing group (N = 24) were allowed to pair for either 48hr or 2 weeks. This resulted in 12 strong pairs (see section on Formation of Pairs for more information on how pairs were determined). To control for the effects of the testing environment, animals who exhibited no pairing behavior were included as controls (see Table 1). To control for the possibility that the animals that did not pair were deficient in some way, an additional 12 subjects (6 of each sex) were derived from same-sex aviaries and not given the chance to pair. Due to tissue damage, 1 paired male and 1 control male were excluded from the analyses (see Table 1 for final animal numbers). All subjects, regardless of pairing group, were separated from their parents at day 50 post-hatching and housed in same-sex aviaries until the start of the experiment. Subjects appeared to be in good health and had not previously formed a pair relationship. Animals were maintained on a timed 12:12hr light—dark cycle in a temperature (24°C) and humidity (50%) controlled room. Seed and water were provided *ad libitum*. Animals were also supplemented with hard-boiled chicken egg and calcium-enriched grain (Simple System Breeder Crumb 5-Day Product, The Bird Care Company) twice per week.

Housing

During pairing tests, subjects were housed in 91.4 ×76.2 × 76.2 cm observation cages. Each cage contained a water dish, food dish, grit box, perches, four empty nest boxes, and nesting materials.

Formation of Pair Relationships

Four birds of each sex were placed into aviaries and allowed to pair for two time periods: either 48hr or 2 weeks. These two time points were chosen because most animals pair within 48hr (Silcox & Evans, 1982), and 2 weeks is enough time to form a solid pair relationship

(Tomaszycki & Adkins-Regan, 2006) without the confound of parenting behavior. In no cases were eggs or nestlings produced. Subjects from the unpaired and 2 paired groups were derived from a total of 8 cohorts. Birds exposed to the pairing paradigm were observed for 30 minutes on the first day: 15 minutes between 11:00 and 12:00hr and then again between 13:00 and 15:00hr. This was to determine if pairing occurred on the first day, since pairing can occur quickly (Silcox & Evans, 1982). We then observed birds for 15 minutes on the second and third days between 11:00 and 12:00hr. Overall pairing and courtship behaviors (see Table 2) were recorded as in our earlier work (Pedersen & Tomaszycki, 2012; Smiley, Vahaba, & Tomaszycki, 2012; Tomaszycki & Adkins-Regan, 2005; Vahaba, Lacey, & Tomaszycki, 2013). Observers recorded behaviors using a stopwatch and premade observation sheets. All observers (N=6) were trained to a high degree of inter-observer reliability (>95%) before the start of observations and we periodically checked inter-observer reliability to insure that this high degree was maintained.

Pairing status was assessed using an association index, in which a subject was considered paired if more than 75% of the subject's pairing behaviors were with one partner relative to other opposite-sex animals (Mabry, Streatfeild, Keane, & Solomon, 2011). Only animals that paired by day 2 and were paired with the same animal on day 3 were used in subsequent analyses. Once paired, subjects in the 2 week group were observed three times per week on Monday, Wednesday and Friday, between 11:00 and 12:00hr, until 2 weeks post-pairing. Only animals that maintained the same partner throughout the 2 weeks were included in the analyses (see Table 1 for animal totals).

Brain Collection

Immediately after observations on the last day of testing for each pairing group, brains were collected via rapid decapitation, frozen in cold methyl-butane, and stored at -80°C. Control animals (the ones housed in same-sex aviaries) were not observed and their brains were collected using the same protocol. Brains were then sectioned coronally at 20µm on a Leica cryostat and mounted directly onto SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA).

Probe Preparation

Probes were derived from published sequences (VT: Genbank:XM_002190047; MT: Genbank:DQ213341). These sequences are highly homologous (81-85%) to corresponding genes in the chicken. MT was obtained as a glycerol stock from Erich Jarvis's laboratory (Duke University). Plasmid DNA was isolated using Wizard Plus Minipreps (Promega, Madison, WI). To obtain T3 (anti-sense) & T7 (sense) probes, we used a Qiagen Maxi Prep kit (Valencia, CA), and linearized the templates using the restriction enzymes XhoI (T3, anti-sense) and NotI (T7, sense). A cold transcription reaction was performed to confirm product quality of the correct size.

We developed primers for VT using the NCBI primer tool (Forward = TGCTCTCCGGCAGTGCATGC; reverse = GCTGTCCATGGCGCACGTCT, resulting in a 235 bp product that was located on exon 2, including base pairs 129-144 of the total VT sequence), and conducted reverse-transcribed PCR (#12574-035, Invitrogen, Carlsbad, CA) per manufacturer's instructions on isolated RNA to obtain the DNA. According to the NCBI database, the resulting VT sequence had no alignment with MT, nor did it have the potential for other off-target effects. The resulting products from both MT and VT were sequenced using central facilities (MT: Michigan State University; VT: Wayne State University) and specificity

for zebra finch MT and VT were confirmed using the BLASTn tool on the NCBI website. Both probes were prepared using Roche Applied Science DIG RNA Labeling kits according to manufacturer's instructions (catalog # 11175025910, Indianapolis, IN).

Dot Blot Assay

To determine ideal probe concentrations, we conducted a dot blot assay as in previous work (Patel et al., 2012). Briefly, the probes were serially diluted (1:10, 1:100 and 1:1,000) in RNase-free water and spotted onto a charged nylon membrane (Millipore, Billerica, MA, USA). DIG incorporation was assessed after incubating with a 1:1500 dilution of alkaline phosphatase conjugated anti-digoxigenin. Probe signal was developed in alkaline phosphatase buffer (0.1M NaCl, 0.05 MgCl2, 0.1 M Tris pH 9.5, 0.1% Tween 20) with NBT/BCIP (Roche Applied Science, Indianapolis, IN). The highest concentration that yielded a detectable spot was then used on trial tissue. We confirmed that this concentration (1:1000 for both MT and VT) yielded specific staining with no background (see Figure 1).

In Situ Hybridization

Fluorescence *in situ* hybridization was then conducted as in our earlier work (Thompson, Dzubur, Wade, & Tomaszycki, 2011; Tomaszycki & Dzubur, 2013). Slides were fixed with 3% paraformaldehyde, acetylated, dehydrated, and air dried. Hybridization occurred at 55°C overnight with 1:1000 concentration of MT or VT probe in hybridization buffer. After a series of washes, slides were incubated in 0.3% hydrogen peroxide in TNT buffer for 10 minutes, and blocked in TNB Buffer for a half hour. Slides were then incubated in the secondary antibody (1:100, Anti-DIG-POD, #11207733910, Roche Applied Science, Indianapolis, IN) for 2hr, followed by 30 minutes in a tyramide-conjugated fluorophore (1:100, TSA kit #22, Alexa 488,

Invitrogen, Carlsbad, CA). Finally, slides were cover-slipped with Slow Fade (#S36937, Invitrogen, Carlsbad, CA), cured overnight, and sealed with nail polish.

Quantification

Slides were analyzed using a Nikon Eclipse 80i microscope and Nikon Elements Software (AR 3.0). An observer (EML), who was blind to pairing groups and sex, quantified the number of cells (by hand, using the program which allows you to keep track of your counts) in each section in each brain area within a 256 x 196 µm box. A standard threshold was applied to all slides; only cells with fluorescence levels above this threshold were considered stained for MT or VT mRNA. We used a conservative threshold, since we used a tyramide-conjugated fluorophore that could result in amplification of background staining. For MT mRNA, the number of cells in the PVN was measured. For VT mRNA, the PVN and BSTm were measured. These regions were located using adjacent slides stained with thionin. On average, 4 sections (representing both left and right hemispheres) were quantified per animal.

Statistical Analysis

All data were analyzed using SPSS Version 19 (SPSS Inc. 2010, Chicago, IL). The mean number of cells expressing MT and VT for each subject was log-transformed to normalize the data. The effects of Sex and Treatment (independent variables) on the number of cells expressing MT and VT mRNA in the PVN and BSTm (dependent variables) were analyzed using a Sex X Treatment MANOVA with Bonferroni corrections where appropriate. Effect sizes ($\eta 2$) are also reported.

The relationship between pairing and courtship behaviors and MT and VT mRNA was analyzed using multiple linear regressions. Behavioral data were averaged across days and then

log-transformed to achieve normality. MT and VT cell numbers were averaged across each hemisphere in each section and similarly log-transformed. To test the ability of behaviors to predict the nonapeptide expression, MT and VT in the PVN (or VT in the BSTm) were entered as dependent variables, and behaviors (directed singing, clumping, nesting, and allopreening) were entered as independent variables in the multiple linear regression. Copulations and tail quivers occurred too infrequently to permit inclusion in the analyses. The multiple linear regressions were run for each sex separately. Stepwise, backward, and forward multiple linear regressions were run; only models with the highest adjusted R^2 values are presented, as in earlier work (Alger et al., 2011). All models and associated p-values are reported in Tables 3 and 4.

Results

Effects of Sex and Pairing on the Number of Cells Expressing MT and VT mRNA in the PVN and BSTm

The overall Wilks' Lambda revealed a main effect of Pairing ($F_{9,76}$= 14.14, P < 0.001, η^2 = 0.55) and Sex ($F_{3,31}$= 5.71, P = 0.003, η^2 = 0.36), but no Sex X Pairing interaction ($F_{9,76}$< 1, η^2 = 0.08). The effect of Pairing was statistically significant for MT and VT in the PVN (MT: $F_{3,33}$ = 70.48, p < 0.001, η^2 = 0.87; VT: $F_{3,33}$ =15.13, p < 0.001, η^2 = 0.58; see Figures 2 and 3), as well as VT in the BSTm ($F_{3,33}$ = 3.36, p = 0.03, η^2 = 0.23; see Figure 4). Specifically, in the PVN, both paired groups (48hr and 2 weeks) had a higher number of cells expressing MT and VT than did either of the control groups. The paired groups did not differ significantly from each other, nor did the control groups. In the BSTm, animals paired for 48hr had a higher number of cells expressing VT than did animals that were not given the opportunity to pair.

The overall sex difference proved only to be significant for VT mRNA in the BSTm ($F_{1,33}$ =18.14, $p < 0.001$, $\eta^2 = 0.36$; Figure 4). Regardless of pairing status, males had a higher number of cells expressing VT in the BSTm than did females. There were no sex differences in the PVN (MT: $F_{1,33}<1$, $\eta^2 = 0.02$, Figure 2 ; VT: $F_{1,33}<1$, $\eta^2 = 0.03$, Figure 3).

Contributions of Behavior to Variations in MT and VT mRNA

Behaviors differentially predicted the number of cells stained for MT and VT mRNA in the PVN (all results presented in Table 3). In males, directed singing significantly predicted variations in the number of cells expressing MT mRNA. Directed singing and clumping best explained the number of cells expressing VT mRNA in the PVN (see Table 3). In the BSTm, variations in VT mRNA were best explained by a model including singing, allopreening, and clumping (the latter contribution was not statistically significant, see Table 4).

In females, a model including the pairing behaviors clumping and allopreening (the latter was not statistically significant) predicted variations in MT mRNA (see Table 3). No behaviors predicted variability in the number of cells expressing VT mRNA in either the PVN or BSTm (see Table 4).

Discussion

Effects of Pairing and Sex on Nonapeptide mRNA

The present study supports our first hypothesis, that pairing increases nonapeptides in both the PVN and BSTm of zebra finches of both sexes. To our knowledge, this is the first study to demonstrate pairing-related increases in nonapeptide mRNA in any avian model. OTAs decrease pairing in zebra finches (Klatt & Goodson, 2013; Pedersen & Tomaszycki, 2012). Taken together, these studies suggest that nonapeptides play an important role in zebra finch

pairing: nonapeptides affect pairing and, in turn, pairing affects nonapeptides. If this bi-directional relationship is true, then our effects of pairing on VT mRNA are surprising, since earlier work has found no effects of vasotocin antagonists on pairing (Goodson et al., 2004; Kabelik et al., 2009).

In the PVN, pairing, regardless of length, increased the number of cells expressing nonapeptide mRNA. This suggests that forming and maintaining pair-bonds may result in elevated nonapeptide mRNA in the PVN. The mechanisms of pair maintenance receive are not well understood in any species (Aragona et al., 2006). In prairie voles, length of pairing (3-21 days) had no effect on AVP-ir in the paraventricular nucleus of the thalamus (Bamshad, Novak, & de Vries, 1994). Further research on the role of nonapeptides in pair maintenance is definitely warranted.

Pairing for 48hr also increased VT mRNA in the BSTm relative to control animals. Similar results were found in prairie voles; males housed with a female for 48hr had higher levels of VT mRNA in the BSTm than did males housed without one (Wang et al., 1994). Unpaired animals had VT mRNA cell counts in the BSTm that did not differ significantly from paired animals. Exposure to opposite-sex individuals and courtship behaviors could explain these findings. Presenting a male with a female increases cells immunoreactive for both c-Fos (an immediate early gene) and VT (Goodson et al., 2009; Goodson & Wang, 2006). Thus, the BSTm may be more linked to courtship or the very early stages of pairing.

Males, regardless of treatment, had higher VT mRNA in the BSTm than did females, consistent with findings on VT protein expression across many species (De Vries & Panzica, 2006; Kimura, Okanoya, & Wada, 1999) and VT mRNA in the chicken, in which females

expressed virtually no VT mRNA in the BSTm (Jurkevich, Barth, & Grossmann, 1997). Thus, the present study extends these findings to a sex difference in VT mRNA in the BSTm of the zebra finch.

Pairing-related Behaviors Predict Variations in Nonapeptide mRNA

We conceptualized pairing as a 3-step process: male courtship, female mate choice, and clumping (direct physical contact) in both sexes. We used multiple linear regressions to determine which behaviors best explained variations in nonapeptide mRNA within each sex.

Males: Singing Predominantly Predicts Variations in Nonapeptide mRNA

We hypothesized that directed singing would explain variations in nonapeptide mRNA in males. This was indeed the case. Directed singing was included in all 3 models (MT and VT mRNA in the PVN, and VT mRNA in the BSTm). As stated above, the link between VT in the BSTm and male singing is no surprise—this link has been found when examining VT protein in this same region. The link between directed singing and both VT and MT mRNA in the PVN is, to our knowledge, novel. The functional consequences of this singing-related increase in nonapeptide mRNA in the PVN of males warrants further investigation.

Singing was the only behavior that explained variations in MT mRNA in the PVN of males. This could indicate that courtship, and not pairing, increases MT mRNA in males. Pairing behaviors did, however, explain variations in VT mRNA in both the PVN and BSTm, but only one pairing behavior was significant in each model. In zebra finches, knockdown of VT production in the septum (which receives dense projections from the BSTm) decreases social contact in males (Kelly et al., 2011), which could suggest a role for VT in this component of

pairing. Nonetheless, in males, our findings support our hypothesis that courtship singing is highly important for changes in the nonapeptide system.

Females: Pairing-related Behaviors Predict Variations in Mesotocin mRNA

As predicted, findings in females differed from those in males. First, directed singing received by females did not explain variations in nonapeptide mRNA. We hypothesized that the second step in the pairing process would be female mate choice through song perception. However, it is likely that song *quality* is just as important as is song *quantity* (measured in the present study), as our previous research suggests (Tomaszycki & Adkins-Regan, 2005).

Instead, pairing behaviors in females were more predictive of variations in nonapeptide mRNA, specifically MT mRNA, than were courtship behaviors. A model including clumping and allopreening predicted variations in MT mRNA in the PVN of females. This suggests that pairing may be more important for changes in the mesotocin system of females than of males.

In contrast, no behaviors predicted variations in VT mRNA in either brain region of females. The effect in the BSTm may not be so surprising (although there were no significant Sex X Treatment interactions), since VT mRNA in females was low. However, the findings in the PVN are puzzling, since there were no sex differences in VT mRNA in this region. It is possible that behaviors, not quantified in this study, account for these effects.

Methodological Considerations

Previous studies have examined VT-ir in the BSTm of male zebra fiches. Such studies have documented more cells expressing the VT protein in the BSTm than what we present here. There are multiple reasons for these discrepancies. First, studies of VT-ir in male zebra finches have documented VT-ir numbers in the BSTm that range from 15-150 (Goodson et al., 2009;

Kabelik, Morrison, & Goodson, 2010; Kimura et al., 1999), which suggests that there is tremendous variability in the number of VT-ir cells reported. In one of these studies (Kabelik et al., 2010), each measurement consisted of the total number of cells in both hemispheres, whereas here, we present the average across one counting frame within one hemisphere. Furthermore, we sectioned brains at 20 µm, whereas previous studies have sectioned brains at 40 µm (Kabelik et al., 2010). Thicker sections can lead to a greater numbers of cells. Finally, there is a vast literature that documents mismatches between mRNA and protein expression (Chen et al., 2002; Greenbaum, Colangelo, Williams, & Gerstein, 2003; Press et al., 2008; Wada et al., 2006). Indeed, studies in yeast have found up to a 20-fold difference in the amount of protein and its corresponding mRNA (Gygi, Rochon, Franza, & Aebersold, 1999).

Nonetheless, our differences between the sexes match those of previous studies examining VT protein expression in the BSTm (Kabelik et al., 2010), and suggest the same correlation between VT in the BSTm between males who court and those who do not (Goodson et al., 2009). Thus, despite differences in numbers, our correlations between VT and behavior are similar to studies of VT protein expression in this region. Future studies should employ testing paradigms from the present study and measure VT and MT protein expression to determine the functional outcomes of these changes in mRNA.

We measured mRNA as a proxy for nonapeptide availability. We chose this method because it relies on zebra finch-specific probes. However, there is the possibility that an increase in nonapeptide mRNA does not cause an actual increase in the release of nonapeptides; it could potentially reflect an increase in the accumulation of nonapeptides in the regions we examined (Neumann, 2002). Future research should focus on how pairing alters nonapeptide release or

how it affects activity at nonapeptide receptors. Furthermore, while we can correlate pairing with nonapeptide mRNA, we cannot exclude the possibility that an initial increase in nonapeptide mRNA at the time of aviary formation facilitated pairing. Unfortunately, it is impossible to measure nonapeptide mRNA at two time-points in the same animal.

Finally, we chose a more naturalistic paradigm, which is good for investigating natural choice, but limits experimental control. Pairing is a complex process that involves a wide variety of behaviors. While we have attempted to associate particular behaviors with variations in nonapeptide mRNA in particular brain regions, we do not know the extent to which each behavior is necessary or sufficient to account for our observed effects. Future research could test this using more controlled experimental conditions to separate out singing, song perception, and pairing behaviors.

Conclusions

In summary, our results provide strong evidence that pairing increases nonapeptide mRNA in the PVN and BSTm of adult zebra finches of both sexes, but that different behaviors predict variations in nonapeptide mRNA within each sex. In males, singing predicted variations in nonapeptide mRNA, whereas in females, pairing behaviors predicted variations in MT mRNA only. Therefore, although pairing caused similar increases in nonapeptide mRNA in both sexes, these increases may be driven more by courtship in males and by pairing in females. Our research contributes a further understanding of the role of nonapeptides in monogamous pair relationships.

Table 2.1. Number of subjects, by treatment group and sex, included in a study of the effects of pairing on mesotocin and vasotocin mRNA in adult zebra finches.

	2 week	**48hr**	**Unpaired**	**Control**
Male	6	5	4	5
Female	6	6	3	6

Table 2.2. Pairing and courtship behaviors observed in adult zebra finches of both sexes.

Behavior	**Male- or Female-Typical**	**Duration or Frequency**	**Description**
Singing	Male	Duration	Song directed at a female
Clumping	Both	Duration	Direct physical contact
Allopreening	Both	Frequency	Mutual grooming
Nesting	Both	Duration	Sharing a nest box
Tail Quiver	Female	Frequency	Rapid shaking of tail feathers
Copulation	Male	Frequency	Mating

Table 2.3. Regression models of courtship and pairing behaviors that explained variations in mesotocin (MT) and vasotocin (VT) mRNA in the paraventricular nucleus of the hypothalamus (PVN)of zebra finches of both sexes.

Table 3				
mRNA	Model Statistics	Variables in model	Beta	p-Values
Males Only				
MT mRNA	Adjusted R^2=0.74, $F_{1, 13} = 40.08$, $p < 0.0001$	Directed Singing	0.87	0.0001
VT mRNA	Adjusted R^2=0.65, $F_{1, 12} = 14.19$, $p = 0.001$	Directed Singing	0.67	0.002
		Clumping	0.4	0.027
Females Only				
MT mRNA	Adjusted R^2=0.39, $F_{1, 11} = 5.43$, $p = 0.021$	Clumping	0.99	0.008
		Allopreening	-0.55	0.107
VT mRNA	No significant models returned	(none)		

Table 2.4. Regression models of courtship and pairing behaviors that explained variations in vasotocin (VT) mRNA in the bed nucleus of the stria terminalis (BSTm) of zebra finches of both sexes.

Table 4				
mRNA	Model Statistics	Variables in model	Beta	p-Values
Males Only				
VT mRNA	Adjusted R^2=0.45, $F_{1, 10} = 4.90$, $p = 0.021$	Directed Singing	0.54	0.024
		Clumping	-0.60	0.075
		Allopreening	0.73	0.036
Females Only				
VT mRNA	No significant models returned	(none)		

Figure 2.1. Examples of Mesotocin (MT) and Vasotocin (VT) mRNA in the paraventricular nucleaus of the hypothalamus (PVN) of adult male and female zebra finches paired for 48hr or 2 weeks. Pictures were taken at low (40X) maginification to demonstrate specificity of staining. White outlines indicate the boundaries of the PVN.

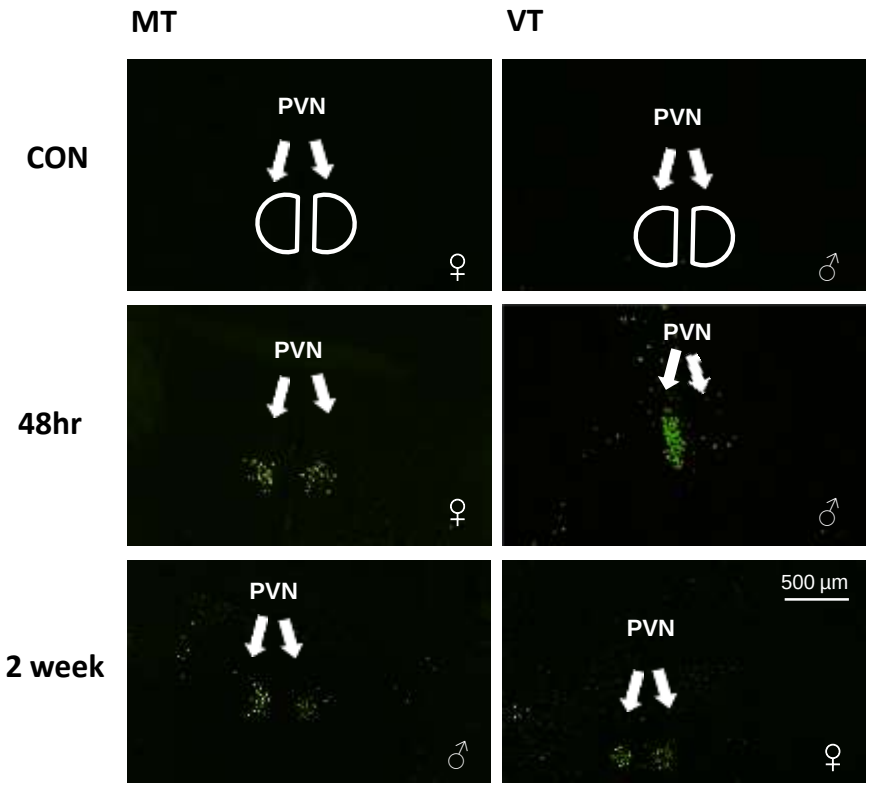


Figure 2.2. Mesotocin (MT) mRNA in the PVN of paired and unpaired adult zebra finches of both sexes. A) Examples of MT mRNA in the paraventricular nucleus of the hypothalamus (PVN) of adult male and female zebra finches paired for 48hr, 2 weeks, or unpaired (control). 400X magnification. B) The mean number of cells stained for MT mRNA in the PVN by pairing group and sex. Animals who paired (both for 48hr and 2 weeks) had a higher number of cells expressing MT mRNA in the PVN than did animals who did not pair (unpair) or were not given the chance to pair (control). There were no sex differences. *indicates significant difference from both paired groups at the $p < 0.05$ level.

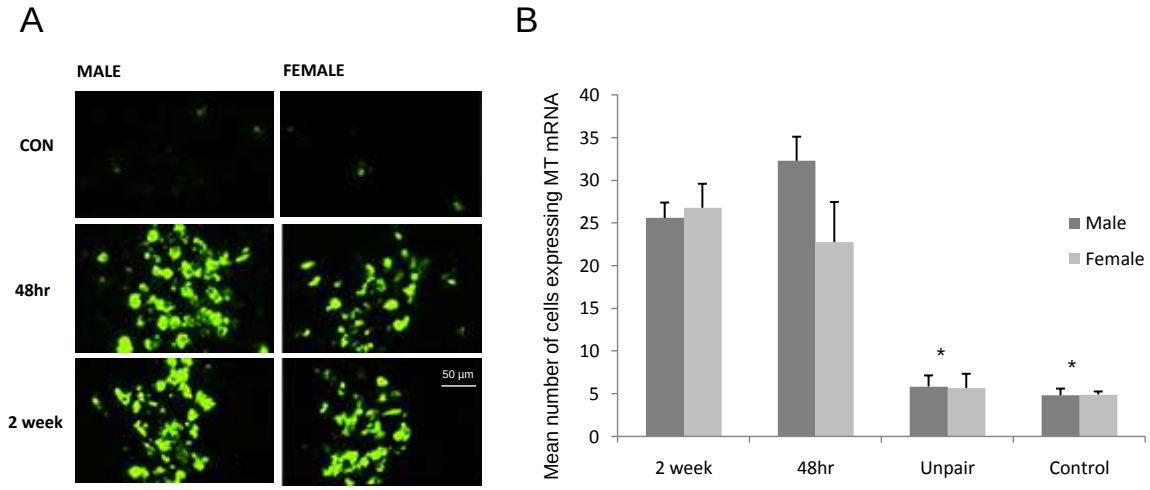


Figure 2.3. Vasotocin (VT) mRNA in the PVN of paired and unpaired adult zebra finches of both sexes. A) Examples of VT mRNA in the paraventricular nucleus of the hypothalamus (PVN) of adult male and female zebra finches paired for 48hr, 2 weeks, or control. 400X magnification. B) The mean number of cells stained for VT mRNA in the PVN by pairing group and sex. Animals who paired (both for 48hr and 2 weeks) had a higher number of cells expressing VT mRNA in the PVN than did animals who did not pair (unpair) or were not given the chance to pair (control). There were no sex differences. *indicates significant difference between groups at the $p < 0.05$ level.

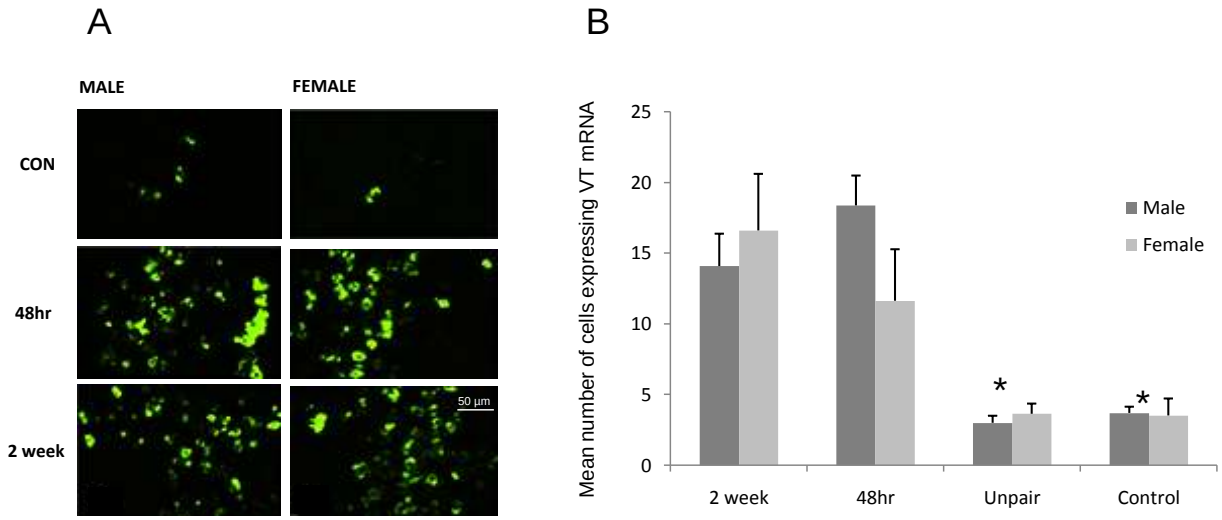
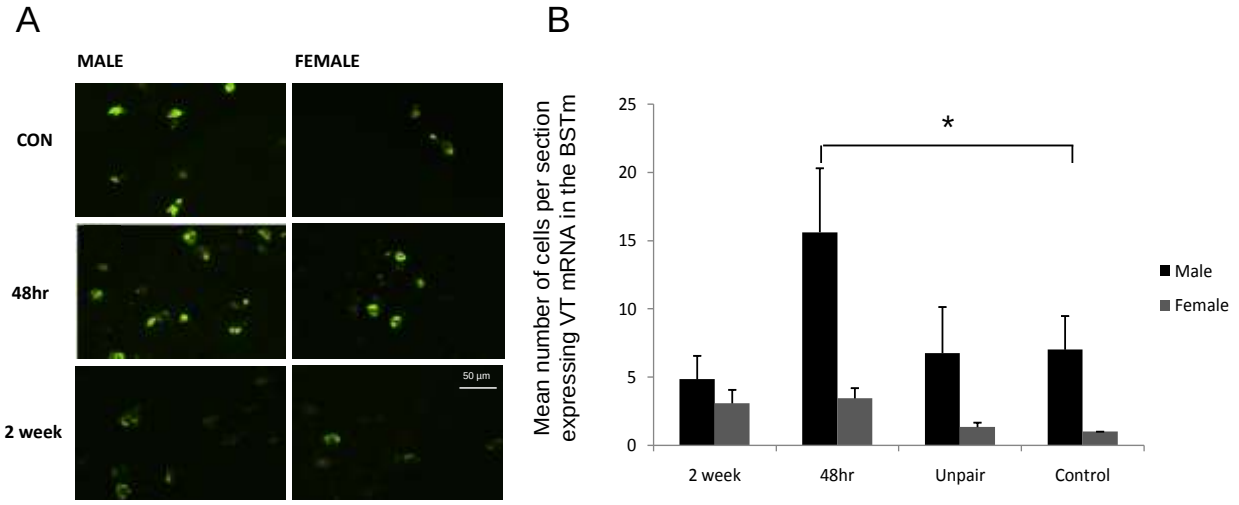


Figure 2.4. VT mRNA in the BSTm of paired and unpaired adult zebra finches of both sexes. A) Examples of VT mRNA in the medial bed nucleus of the stria terminalis (BSTm) of adult male and female zebra finches paired for 48hr, 2 weeks, or control. 400X magnification. B) The mean number of cells stained for VT mRNA in the BSTm by pairing group and sex. Animals paired for 48hr had a significantly higher number of cells expressing VT mRNA than did control animals (not given the opportunity to pair).*indicates significant difference between groups at the $p < 0.05$ level.



CHAPTER 3 - THE ROLE OF CATECHOLAMINES IN COURTSHIP AND PAIRBINDING BEHAVIOR OF MALE AND FEMALE ZEBRA FINCHES

The catecholamine, dopamine (DA), plays a vital role in affiliative behaviors. Affiliative behaviors in animals, such as grooming and tactile contact (Starr & Starr, 1986; Dunbar, 2010), positive interactions (Odendaal & Meintjes, 2003) and viewing images of conspecifics (Saif et al., 2013) have been shown to increase catecholamine levels. Therefore, it is likely that catecholamines also play a role in the formation of social relationships.

DA has been implicated in reward (Wise & Rompre, 1989; Berridge & Robinson, 1998; Noble, 2000; Shultz, 2010) and sexual behavior (Melis & Argiolas, 1995; Pfaus, Kippin, & Centeno, 2001; Dominguez & Hull, 2005). DA receptors are widely distributed throughout the limbic system, a neural circuit strongly implicated in reward (Routtenberg, 1968; Missale et al., 1998; McClure et al., 2004). Beyond the role of DA in sexual activity, DA also facilitates the formation of a partner preference in female prairie voles in the absence of mating (Gingrich et al., 2002), indicating that DA is involved in both the social and sexual aspects of pair bonding. DA also facilitates pair bonding in male prairie voles (Aragona & Wang, 2009).

DA has also been associated with courtship behavior, the first step in the pairing process, in birds. DA levels are higher in male zebra finches after they engage in directed singing, an important courtship behavior (Bharati & Goodson, 2006; Sasaki et al., 2006), and experimentally decreasing DA levels results in a decrease in directed signing and other courtship displays in males (Harding, 2004; Rauceo et al., 2008). Birdsong is a complex vocal behavior learned during early life in a process that is analogous to the learning of human speech (Marler, 1970). Like speech, singing is a social behavior and is used by male zebra finches to communicate to the

larger social group — which can include up to 200 conspecifics (Catchpole & Slater, 1995, Zann, 1996). Zebra finch singing can be divided behaviorally into two types: 'directed' singing is highly aroused, is aimed at another bird, and often occurs during the courtship dance, whereas 'undirected' singing occurs when a finch is either alone or not orienting toward any other bird in particular (Hessler & Doupe, 1999).

Catecholamines are present in brain areas controlling song in male songbirds. TH-ir fibers are present in song areas HVC (letter based name), lateral magnocellular nucleus of the anterior nidopallium (LMAN), robust nucleus of the arcopallium (RA) and Area X of male song birds (Appeltants, Ball, & Balthazart, 2001; Soha, Simizu, Doupe, 1995, Bottjer, 1993; Castelino and Ball, 2005). DSP-4, a specific noradrenergic neurotoxin, significantly reduces the level of DBH-ir in HVC and RA and reduces TH-ir levels in Area X (Castelino and Ball 2005). DBH fibers have also been found in the VTA and SNc, innervating Area X (Castelino, Diekamp et al. 2007). Therefore, it is highly likely that catecholamines mediate song production in the male zebra finch.

Catecholamines may further promote social behavior and male song in reward systems, such as the caudal portion of the VTA (VTAc). In the zebra finch, DBH-positive cells are abundant in the LoC, the site of NE synthesis, as well as regions associated with learning and memory, reward and motivation (e.g. VTA and SNc); Mello, Pinaud et al. 1998; Gale and Perkel 2006). TH-ir neurons in the VTAc positively correlate with the degree of sociality and monogamy in finch species (Goodson, Kabelik, Kelly, Rinaldi, & Klatt, 2009). Furthermore, male singing is positively correlated with TH-ir in the VTAc (Alger, Juang et al. 2011). There is an increase in ZENK in the VTA of males who exhibit singing behavior compared to those who

are silent (Lynch, Diekamp, and Ball, 2008). TH density in the VTA appears to modulate the demonstration of courtship behavior in males (Goodson, Kabelik et al. 2009) and increases when courtship is reciprocated by a partner (Alger, Juang et al. 2011). The number of songs produced by a male finch is positively correlated with the percentage of TH-positive neurons expressing FOS, an IEG, in the VTAc (Goodson, Kabelik et al. 2009). Courtship reciprocation is also related to TH density in the (VMH), an area in the "social behavior network" (Newman, 1999; Goodson 2005). Taken together, this evidence suggests that signing in males, an important courtship behavior, is mediated by catecholamines and is a rewarding behavior.

Hearing song has been shown to affect catecholamines in the brain of female zebra finches. In female white-throated sparrows, playback of male song significantly increases the phosphorylation of tyrosine hydroxylase (TH, the rate limiting step in catecholamine synthesis) in the NCM within 15 min (Matragrano et al., 2012), suggesting that hearing song rapidly engages the catecholamine system (Maney, 2013). Song-exposed females also have increases in co-localization of TH and ZENK in the LoC, where NE is synthesized (Lynch, Diekamp et al. 2012). Taken together, catecholamine expression in the NCM and CMM is important for song perception, and therefore mate choice, in female zebra finches.

The preponderance of evidence supports a role of catecholamines in courtship in males and female perception and choice. Most studies of pair bonding in birds have emphasized short-term interaction in a force-choice paradigm (Immelmann et al., 1991; Appeltants, Del Negro et al. 2002; Vyas, Harding et al. 2008; Goodson, Kabelik, Kelly, Rinaldi, & Klatt, 2009). Thus, it remains unclear how catecholamines affect the formation of a pair bond in a naturalistic setting. The present study investigated the role of forming a monogamous pair bond on catecholamine

protein expression (as measured by TH-ir). ZENK-ir was be used as a proxy measure of neuronal activation in the areas of interest. We analyzed song regions [HVC, RA, LMAN, and Area X], reward associated areas that project to the song system [VTA and SNc], auditory regions [NCM and CMM], and an area in the social behavior network [VMH]. These areas were chosen to determine potentially differential mechanisms of catecholaminergic action in song, song perception, and social behavior.

Method

Subjects

Subjects were chosen from adult wild-type zebra finches (*Taeniopygia guttata*) raised in social aviaries. All subjects, regardless of treatment group, were separated from their parents at day 50 post-hatching and housed in same-sex aviaries until the start of the experiment. All subjects were analyzed for good health and have not previously formed a pair relationship. Animals are maintained on a timed 12:12hr light—dark cycle in a temperature (24°C) and humidity (50%) controlled room. Seed and water are provided *ad libitum.* Animals are also supplemented with hard-boiled chicken egg and calcium-enriched grain (Simple System Breeder Crumb 5-Day Product, The Bird Care Company) twice per week.

Subjects in the pairing group (N = 12) were allowed to pair for 48hr. This continued until we had obtained 12 strong pairs in each group (see section on *Formation of Pairs* for more information on how pairs were determined). To control for the effects of the testing environment, animals who exhibited no pairing behavior were included as controls in an unpaired group. To control for the possibility that the animals that did not pair were deficient in some way, an

additional 12 subjects (6 of each sex) were obtained from same-sex aviaries and not given the chance to pair.

Housing

During pairing tests, subjects were housed in 91.4 ×76.2 × 76.2 cm aviaries. Each aviary contained a water dish, food dish, grit box, perches, four empty nest boxes, and nesting materials.

Formation of Pair Relationships

Four birds of each sex were placed into aviaries and allowed to pair for 3 days. This time point was chosen because most animals pair within 24hr (Silcox and Evans, 1982). Subjects were derived from 6 cohorts. Birds exposed to the pairing paradigm are observed for 30 minutes on the first day: 15 minutes between 11:00 and 12:00hr and then again between 13:00 and 15:00hr. This is to determine if pairing occurred on the third day, since pairing can occur quickly (Silcox and Evans, 1982). We then observed birds for 15 minutes on the second between 11:00 and 12:00hr. Overall pairing and courtship behaviors are recorded as in our previous work (Pedersen and Tomaszycki, 2012; Smiley et al., 2012; Tomaszycki and Adkins-Regan, 2005). Observers record behaviors using a stopwatch and premade observation sheets. All observers (N=2) have been trained to a high degree of inter-observer reliability (>95%) before the start of observations, and maintained this degree of reliability throughout the study.

To determine whether or not subjects were paired, we used an association index, in which a subject was considered paired if more than 75% of the subject's pairing behaviors were with one partner relative to other opposite-sex animals (Mabry et al., 2011). Only animals that paired on day 1 and were confirmed as paired on day 3 with the same individual were used in

subsequent analyses. Birds were considered to be unpaired if they spent less than 20% of their time engaged in courtship behavior over the 3 observation days.

Brain Collection

Immediately after observations on the last day of testing for each pairing group, birds were sacrificed via rapid decapitation and their brains were rapidly extracted, frozen in cold methyl-butane, and stored at -80°C. The brains of control animals (the ones housed in same-sex aviaries), which were not observed, were collected using the same protocol. Brains were then sectioned coronally at 20µm on a Leica cryostat and mounted directly onto SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA).

Immunocytochemistry (ICC)

Slides were brought to room temperature for approximately 10 minutes in order to perform the double-label TH/ZENK ICC. Slides were fixed with 3% paraformaldehyde, briefly rinsed in TBS, and dehydrated in ETOH. This was followed by three rinses in TBS and incubation in 1% H_2O_2 and 0.3% Triton X in TBS for 10 minutes. Slides were then rinsed three times in TBS and blocked for 30 minutes in 2% normal goat serum and 0.3% Triton X in TBS at 37°C. Slides were incubated for 48 hours in primary ZENK antibody (anti-rabbit IgG 1:1000, Santa Cruz Biotechnology, #SC52, Santa Cruz, CA) at 4°C. Slides were rinsed three times in TBS and incubated in biotinylated goat anti-rabbit serum (1:250; Vector Laboratories, Burlingame, CA) in 2% NGS and 0.3% Triton X in TBS for 1 hour. Slides were rinsed three times in TBS and incubated for 1 hour in avidin-biotin peroxidase reagent (ABC Elite Kit, Vector Laboratories, Burlingame, CA). Visualization of ZENK was obtained by incubation of slides for 8 minutes in 3,3'-diaminobenzine (DAB, Sigma Aldrich, St. Louis, MO) in TBS.

Slides were rinsed three times in TBS and blocked in 2% normal goat serum for 30 minutes. Slides were then incubated overnight in primary TH antibody (mouse anti-TH IgG, 1:1000; Chemicon, # MAB318, CITY, STATE) in 2% NGS and 0.3% Triton X in TBS. Slides were rinsed three times in TBS and then blocked in TH secondary (mouse anti-TH IgG, 1:250; Vector Laboratories, Burlingame, CA) in 2% NGS and 0.3% Triton X in TBS for 1 hour. Slides were rinsed three times in TBS and incubated for 1 hour in avidin-biotin peroxidase reagent. After a quick rinse in TBS, visualization of TH was obtained by incubation in Vector SG (Vector Laboratories, Burlingame, CA) in peroxide and TBS for 10 minutes. Slides are then rinsed with distilled water, quickly dehydrated in ETOH, and coverslipped.

Quantification

Regions were quantified using a Nikon Eclipse 80i microscope and Nikon Elements Software (AR 3.0). The observers (KM, WL, and EL), who were blind to treatment groups, quantified the number of cells in each brain area. For cells containing TH, ZENK, or both TH and ZENK, nine brain regions were quantified. These include the song areas: Area X, LMAN, HVC, and RA; the auditory areas CMM and NCM; and areas regulating social behavior and reward, VTA, SNc, and VMH. These regions were located using adjacent slides stained with thionin. On average, 3 sections (representing both left and right hemispheres) were quantified per animal.

Statistical Analysis

All data were analyzed in SPSS v.22 (SPSS Inc. 2013, Chicago, IL). Prior to analysis, the mean number of cells expressing TH-ir, ZENK-ir, and co-localization of TH-ir + ZENK-ir across regions were log-transformed to achieve normality. In order to examine the several behaviors as

repeated measures of courtship, frequency count and duration scales were equated by transforming all behavioral data to z-scores. Two control treatment groups were created: birds that remained unpaired despite the opportunity to pair, and those who were housed in same-sex aviaries and not allowed to pair. Differences in immunoreactivity within each region and behavior were tested in a 3 (immunoreactivity: TH-ir, ZENK-ir, TH+ZENK-ir) x3 (experimental group: Paired, Unpaired, Control) multivariate analysis of variance (MANOVA). Correlations among immunoreactivity measures across brain areas were high (all r = -0.52-0.89), indicating a risk of multicollinearity. Due to the limitations of sample size and multicollinearity across regions, separate MANOVA were run by region (Area X, LMAN, HVC, VMH, VTAr, VTAc, SNc, CMM, and NCM). Bonferroni post-hocs were conducted to follow up on significant effects. Finally, because regions of interest differed between the sexes (i.e. Area X, LMAN, and HVC were only measured in males and NCM and CMM were only measured in females), MANOVAs were run separately for males and females for a total of 15 total models (9 regions in males, and 6 regions in females; Area X, LMAN and HVC were not analyzed in females). Due to an inability to adequately sample tissue, 10 unpaired control females displayed damaged tissue in areas of interest, resulting in an inadequate sample size. Two of the female brains were sectioned improperly. Three of the brains were destroyed during handling. The other brains were misplaced. To allow for multivariate analyses, the control group in the female birds was defined as both unpaired and birds from same-sex aviaries (n = 6). Measures of cell expression in RA were insufficient for statistical analysis and therefore the region was omitted from hypothesis testing.

Results

Association between Regional Expression and Pairing in Males

TH-ir, ZENK-ir, and TH+ZENK-ir in male zebra finches. The effect of Pairing was statistically significant for TH+ZENK-ir ($p = 0.026$) in Area X, but not for TH-ir or ZENK-ir expression alone (see Table 1). As predicted, Bonferroni post hoc tests revealed that the paired group had a higher number of cells co-expressing ADRA2c mRNA and ZENK-ir than the unpaired group (see Figure 2).

However, contrary to our hypotheses, there was no significant relationship between pairing and TH-ir, ZENK-ir, or TH+ZENK-ir in song areas LMAN and HVC (see Table 1, Figures 2 and 3) or VMH, VTAr, VTAc, or SNc (see Table 1, Figures 4 and 5).

Association between Regional Expression and Pairing in Females

TH-ir , ZENK-ir, and TH+ZENK-ir in female zebra finches. The effect of Pairing was statistically significant for ZENK-ir in NCM ($p = 0.002$), but not for TH-ir or TH+ZENK-ir expression (see Table 2, Figure 6 and 7). As predicted, the Bonferroni post-hoc revealed that the paired group had a higher number of fibers expressing ZENK-ir than the unpaired group ($p = 0.002$). However, pairing had no effect in CMM (see Table 2, Figure 6 and 7), which was unexpected.

The effect of Pairing was statistically significant for ZENK-ir and TH+ZENK-ir expression in VMH, but not for TH-ir alone (see Table 2, Figure 6 and 7). The Bonferonni post-hoc supported out hypotheses, such that the paired group had a higher number of fibers expressing ZENK-ir alone ($p = 0.004$) and a lower number of fibers expressing TH+ZENK-ir ($p = 0.006$) than the unpaired group (see Figures 6 and 7).

The effect of Pairing was statistically significant for ZENK-ir in VTAr, such that the paired group had a lower number of cells than the unpaired group ($p = 0.038$; see Table 2, Figures 8 and 9), which was the opposite effect we were expecting. TH-ir and TH+ZENK-ir expression were not significant (see Table 2, Figures 8 and 9)

The effect of Pairing was statistically significant for ZENK-ir and TH+ZENK-ir expression in VTAc, but not for TH-ir (see Table 2, Figures 8 and 9). Contrary to the direction we predicted, the paired group had significantly less cells expressing ZENK-ir ($p = 0.015$) and more cells expressing TH+ZENK-ir ($p = 0.004$) than the unpaired group (see Figures 8 and 9).

Unexpectedly, there was no effect of Pairing in SNc for TH-ir, ZENK-ir, or TH+ZENK-ir (see Table 2, Figures 8 and 9).

Contributions of Behavior to Variations in TH-ir, ZENK-ir, or TH+ZENK-ir

Only statistically significant relationships between pairing and molecular expression were analyzed using linear regression. In Area X of males, none of the behaviors significantly predicted variations in the number of fibers expressing TH+ZENK-ir co-expression (see Table 1). In the NCM of females, clumping and nesting statistically predicted the number of fibers expressing ZENK-ir (see Table 1). In the VMH of females, clumping statistically predicted the number of fibers expressing ZENK-ir, but no behaviors statistically predicted TH+ZENK-ir (see Table 1). In the VTAc of females, nesting statistically predicted the number of fibers expressing TH+ZENK-ir, but no behaviors statistically predicted ZENK-ir (see Table 1). In the VTAr of females, clumping statistically predicted the number of fibers expressing ZENK-ir (see Table 1).

Discussion

TH-ir, ZENK-ir, and co-expression in the male zebra finch

Our findings demonstrate significantly more fibers co-expressing TH-ZENK-ir in Area X of paired than unpaired males. High levels of both noradrenergic (Ball & Balthazart, 2007; Cornil, Castelino et al. 2008; Castelino, Diekamp et al. 2007; Riters & Ball, 2002) and dopaminergic (Appeltants, Ball, & Balthazart, 2001; Soha, Simizu, Doupe, 1995, Bottjer, 1993; Castelino & Ball, 2005) receptors have been found in Area X. This area is active when hearing the bird's own song and is important for online monitoring of song so feedback can be provided to other song nuclei for proper song production (Solis, Brainard et al. 2000). High rates of NE turnover have been found in Area X after exposure to a female (Barclay and Harding 1988), indicating that this area is also important for motivated courtship behaviors, such as directed singing.

Area X is innervated by noradrenergic fibers from the LoC and dopaminergic fibers from the VTA and SNc (Castelino, Diekamp et al. 2007). While a linear regression did not show any correlations between TH-ZENK-ir expression and recorded behaviors, previous work has shown that dopaminergic cells in the VTA are involved in the motivational aspect of zebra finch song (Yanagihara and Hessler, 2006). It is possible that this may be the mechanism contributing to increased TH-ZENK-ir expression in Area X, even though no significant increases were found in the VTA of these birds. Because Area X was the only one of the song areas to show any significant difference in expression, it is likely that the TH-ZENK-ir expression in this area is due to dopaminergic input as opposed to noradrenergic input.

TH-ir, ZENK-ir, and co-expression in the female zebra finch

We hypothesized that female song perception and mate choice would be mediated by catecholamines. In paired females, there was increased expression of ZENK-ir in NCM and

VMH, but a decrease in VTAr, and VTAc. TH-ir alone was not significantly different between groups, but TH+ZENK-ir was lower in VMH and higher in the VTAc of paired than unpaired females.

The NCM is an auditory area important for female song perception (MacDougall-Shackleton, Hulse et al. 1998) and has been found to contain high density of TH-ir fibers (Appeltants, Ball, & Balthazart, 2001). ZENK-ir is a measure of activity and is rapidly induced in the NCM of female zebra finches specifically in response to male zebra finch song, as opposed to other auditory stimuli (Mello, Vicario, et al., 1992). The linear regression revealed that both clumping and nesting behaviors were positively related to increased ZENK-ir in paired females, but not directed singing. This indicates that the NCM may be responsive to female choice, of which clumping is a clear sign (Zann, 1996), which is a direct result of song assessment.

Our findings in the VMH are contradictory, as pairing was associated with increased ZENK-ir, but decreased TH+ZENK-ir expression. In female zebra finches, previous research has found that greater TH-ir densities in VMH were positively related to clumping behavior and partner's overall courtship behavior (Alger, Juang, & Riters, 2011). We found that increased ZENK-ir in the VMH was also positively related to clumping behavior in paired females, which is consistent with previous work. No behaviors were related to the decrease in TH-ZENK-ir in the VMH, so it is possible that this finding may be an anomaly due to small sample size. The VMH is a part of the social behavior network and deserves further study, particularly in female mate choice.

Furthermore, in paired females, decreased levels of ZENK-ir were found in the VTAr and VTAc, but increased levels of TH-ZENK-ir was found in the VTAc. Previous work has shown that the caudal and rostral regions of the VTA have different relationships to behavior, with the VTAc being positively related to directed signing behavior (Goodson, Kabelik, et al., 2009; Alger, Juang, & Riters, 2011) as well as sociality and monogamy in finch species (Goodson, Kabelik, et al., 2009). In paired males, TH-ir density in the VTAr has been related positively to receiving courtship behavior from a partner, but negatively to clumping behavior while the VTAc was positively related to both receiving courtship behavior from a partner and directed singing (Alger, Juang, & Riters, 2011). Our study found a positive relationship between ZENK-ir expression and clumping behavior in the VTAr and a positive relationship between nesting behavior and TH-ZENK-ir in the VTAc. Alger, Juang, & Riters (2011) suggest that DA in VTAc is related to the production of courtship behavior, whereas DA in VTAr may be more closely related to receiving social behavior from a partner. In our study, only VTAc was found to have significant measures of DA expression, which was positively associated with nesting behavior. Nesting behavior involves both partners and is shared activity between bonded partners (Zann, 1996), which would be considered a production of pairing behavior. It may be that female brain areas react differently throughout the formation of the relationship — such that activity in areas associated with social behavior, such as the VMH, may peak during courtship and areas involved in sexual reward, such as the VTA, may peak during the preparation for parenthood (for the purpose of procreation). This could account for the decrease in TH+ZENK-ir in the VMH, while TH+zenk-ir was increased in the VTAc of paired females in our study.

Further study of DA activity in the regions of the VTA is needed to determine if this hypothesis is true in zebra finches.

Conclusion: The role of TH-ir in Zebra Finch Pair bonding

Here we demonstrated that catecholamine expression may mediate courtship and pairing behaviors in the motivational areas of the zebra finch. This intriguing finding has been suggested in studies of other animal models, but has not been validated in a socially gregarious species, such as the zebra finch. For the first time we studied semi-naturalistic pairing and tested sexually dimorphic behaviors and related neuroanatomy, which is rather novel. Based upon natural pairing, increased TH-ZENK-ir co-expression was found in Area X of male finches and the VTAc, while a decrease was found in the VMH of paired females. Increased ZENK-ir levels were found in the VMH and NCM of paired females, which is a proxy measure of activation in these areas. However, decreased levels of ZENK-ir were found in both regions of the VTA, which was not expected. This study did not directly measure activation, however, it is plausible that the pairing process is related to activation in perception and social behavior areas, such as the NCM and VMH, more selectively than general reward areas, such as the VTA.

Future studies should use larger sample sizes and earlier sampling to capture the effect of perception. It is also possible that the ZENK-ir measures may not be an accurate measure of activity in this experimental paradigm. ZENK-ir is a rapid measure of brain activity and peaks at 90 mins for protein expression after exposure to novel stimuli (Cecchi, Riberio, et al., 1999). In our naturalistic paradigm, it is possible that the zebra finches had acclimated to the environment, or the converse, that after 48 hours together as a bonded pair, the couple was preparing for parenthood, which was novel. Little research has been done on the effect these relationship

transitions may have on neurobiology in the zebra finch. This line of research deserves further study, as determining the molecular substrates of social behavior will improve our understanding of the etiology of psychoaffective disorders and possible pharmaceutical intervention.

Table 3.1. Results from MANOVAs tested in a 3 (immunoreactivity: TH-ir, ZENK-ir, TH+ZENK-ir) x 3 (experimental group: Paired, Unpaired, Control) design by region in male zebra finches.

Expression	*df*	*F*	η	*P*
Area X				
TH-ir	2	2.648	0.289	0.108
ZENK-ir	2	3.204	0.33	0.074
TH+ZENK-ir	2	4.865	0.428	0.026*
LMAN				
TH-ir	2	2.024	0.224	0.169
ZENK-ir	2	2.021	0.224	0.169
TH+ZENK-ir	2	1.83	0.207	0.197
HVC				
TH-ir	2	0.43	0.062	0.66
ZENK-ir	2	0.929	0.125	0.419
TH+ZENK-ir	2	1.076	0.142	0.369
VMH				
TH-ir	2	1.599	0.21	0.242
ZENK-ir	2	1.092	0.154	0.367
TH+ZENK-ir	2	1.599	0.21	0.242
VTAr				
TH-ir	2	0.7	0.104	0.516
ZENK-ir	2	2.514	0.295	0.122
TH+ZENK-ir	2	0.7	0.104	0.516
VTAc				
TH-ir	2	1.858	0.236	0.198
ZENK-ir	2	1.72	0.223	0.22
TH+ZENK-ir	2	1.858	0.236	0.198
SNc				
TH-ir	2	1.117	0.147	0.357
ZENK-ir	2	0.231	0.034	0.797
TH+ZENK-ir	2	0.751	0.104	0.491

Note: Significant findings are indicated by an * at ($p < 0.05$).

Table 3.2. Results from MANOVAs tested in a 3 (immunoreactivity: TH-ir, ZENK-ir, TH+ZENK-ir) x 2 (experimental group: Paired, Unpaired) design by region in female zebra finches.

Expression	*df*	*F*	η	*P*
NCM				
TH-ir	1	0.951	0.08	0.35
ZENK-ir	1	15.86	0.59	0.002*
TH+ZENK-ir	1	0.792	0.067	0.393
CMM				
TH-ir	1	0.019	0.002	0.893
ZENK-ir	1	0.021	0.002	0.887
TH+ZENK-ir	1	0	0	0.991
VMH				
TH-ir	1	0.569	0.049	0.466
ZENK-ir	1	13.05	0.543	0.004*
TH+ZENK-ir	1	11.768	0.517	0.006*
VTAr				
TH-ir	1	0.846	0.071	0.377
ZENK-ir	1	5.538	0.335	0.038*
TH+ZENK-ir	1	3.823	0.258	0.076
VTAc				
TH-ir	1	0.846	0.071	0.377
ZENK-ir	1	8.331	0.431	0.015*
TH+ZENK-ir	1	13.017	0.542	0.004*
SNc				
TH-ir	1	1.776	0.139	0.21
ZENK-ir	1	2.32	0.174	0.156
TH+ZENK-ir	1	0.06	0.005	0.81

Note: Significant findings are indicated by an * at ($p < 0.05$).

Table 3.3. Regression models of courtship and pairing behaviors that explained variations in TH-ir, ZENK-ir, and TH+ZENK-ir co-expression in the brain areas of male and female zebra finches.

Brain Area	IR	Model Statistics	Vaiables in model	Beta	p-Values
Males Only					
Area X	TH+ZENK-ir	No significant models returned	(none)		
Females Only					
NCM	ZENK-ir	Adjusted $R^2 = 0.62$, $F(1,13) = 8.02$, $p = 0.008$	Clumping	0.68	0.006
			Nesting	0.46	0.041
VMH	ZENK-ir	Adjusted $R^2 = 0.54$, $F(1,13) = 12.71$, $p = 0.004$	Clumping	0.68	0.004
	TH+ZENK-ir	No significant models returned	(none)		
VTAr	ZENK-ir	Adjusted $R^2 = 0.40$, $F(1,13) = 6.78$, $p = 0.026$	Clumping	0.64	0.026
VTAc	ZENK-ir	No significant models returned	(none)		
	TH+ZENK-ir	Adjusted $R^2 = 0.38$, $F(1,13) = 6.07$, $p = 0.033$	Nesting	0.62	0.033

Figure 3.1. Examples of TH-ir, ZENK-ir, and TH+ZENK-ir in Area X, LMAN, and HVC of adult male zebra finches paired for 48hr, unpaired, or control. 400X magnification.

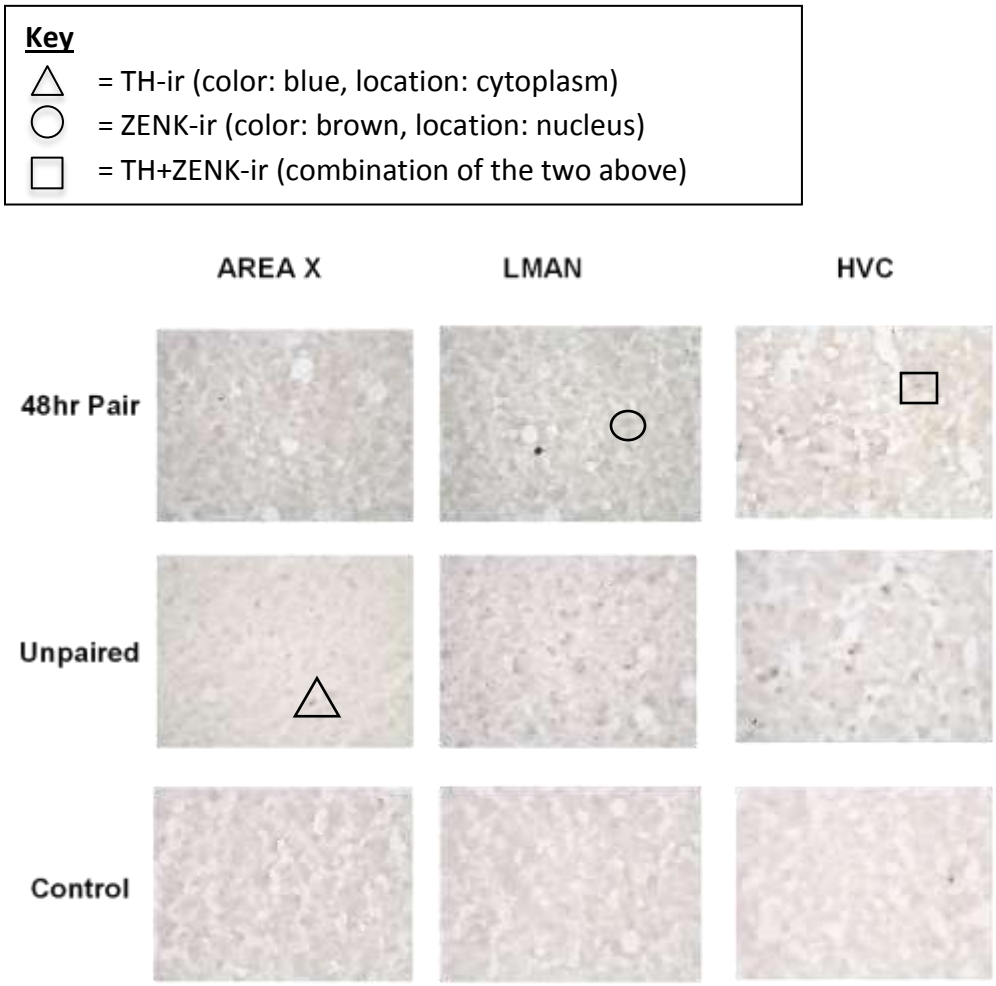
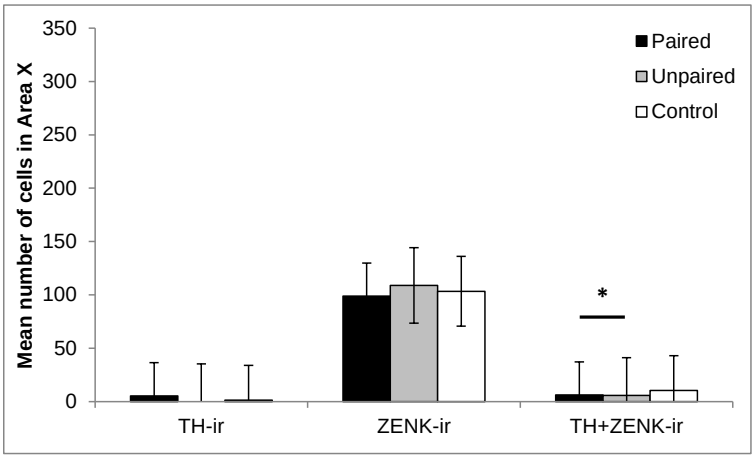
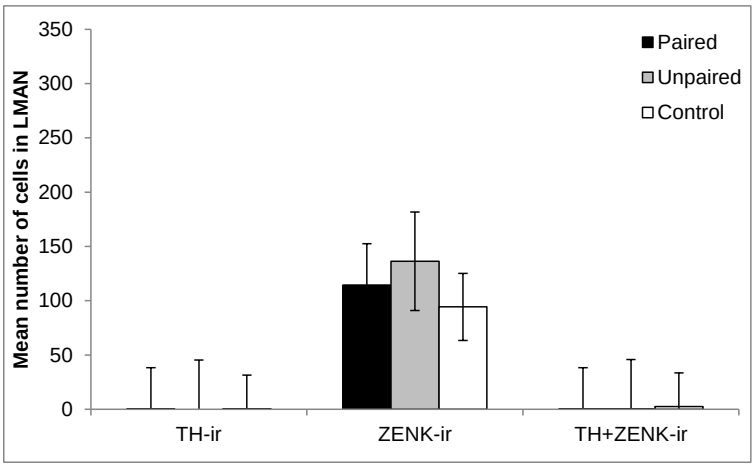


Figure 3.2. Bar graphs represent the mean number of TH-ir, ZENK-ir, and TH+ZENK-ir expressing fibers for Area X, LMAN, and HVC in male zebra finches. Statistical significance is indicated by the * and error bars represent the standard error.





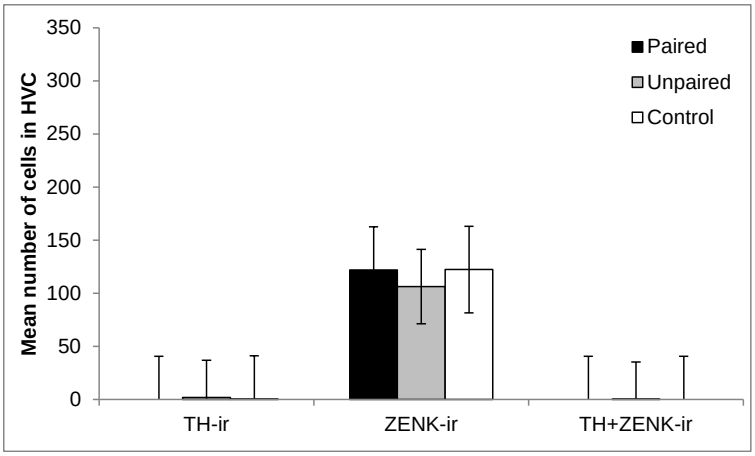


Figure 3.2. Examples of TH-ir, ZENK-ir, and TH+ZENK-ir in VMH, VTAr, VTAc, and SN of adult male zebra finches paired for 48hr, unpaired, or control. 400X magnification.

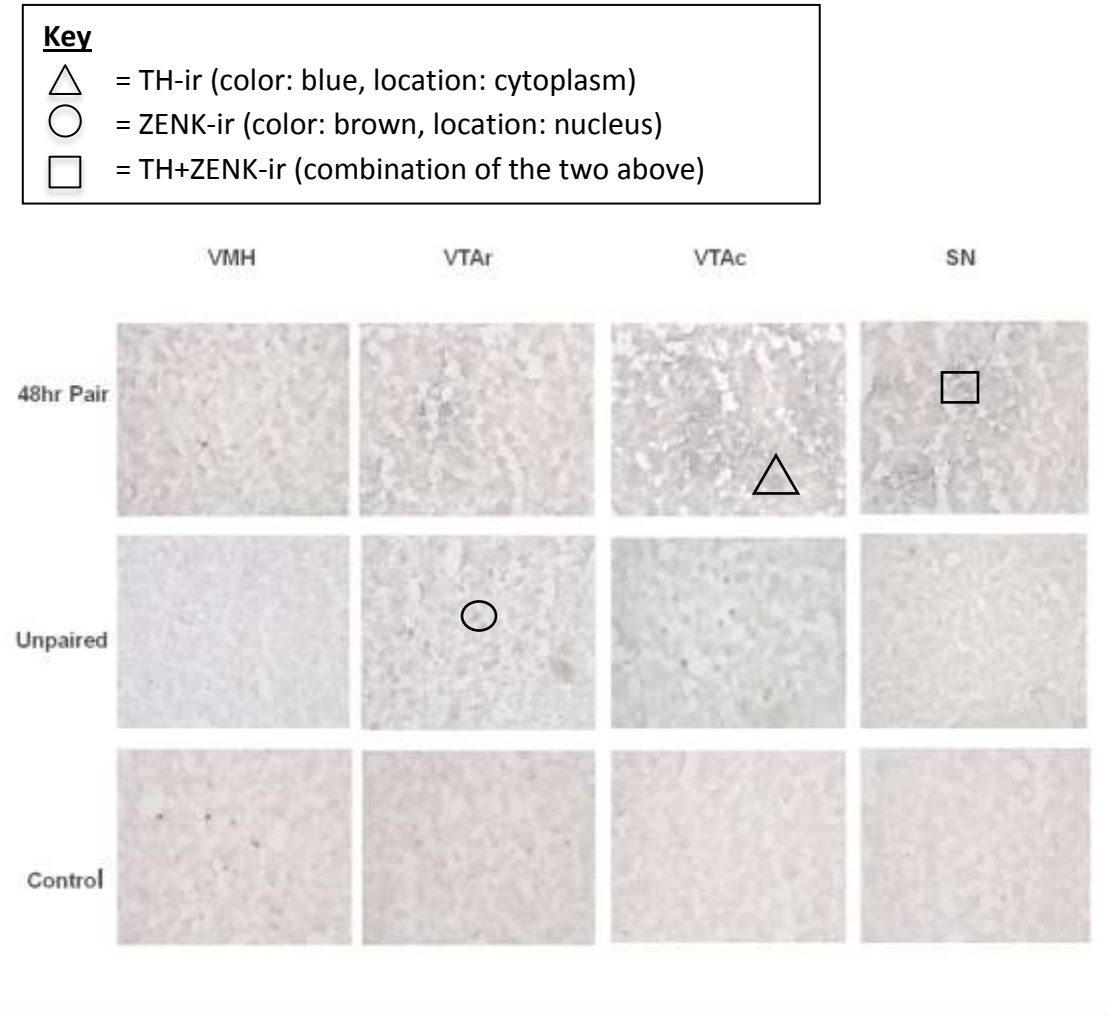


Figure 3.4. Bar graphs represent the mean number of TH-ir, ZENK-ir, and TH+ZENK-ir expressing fibers for VMH, VTAr, VTAc, and SNc in male zebra finches. Statistical significance is indicated by the * and error bars represent the standard error.

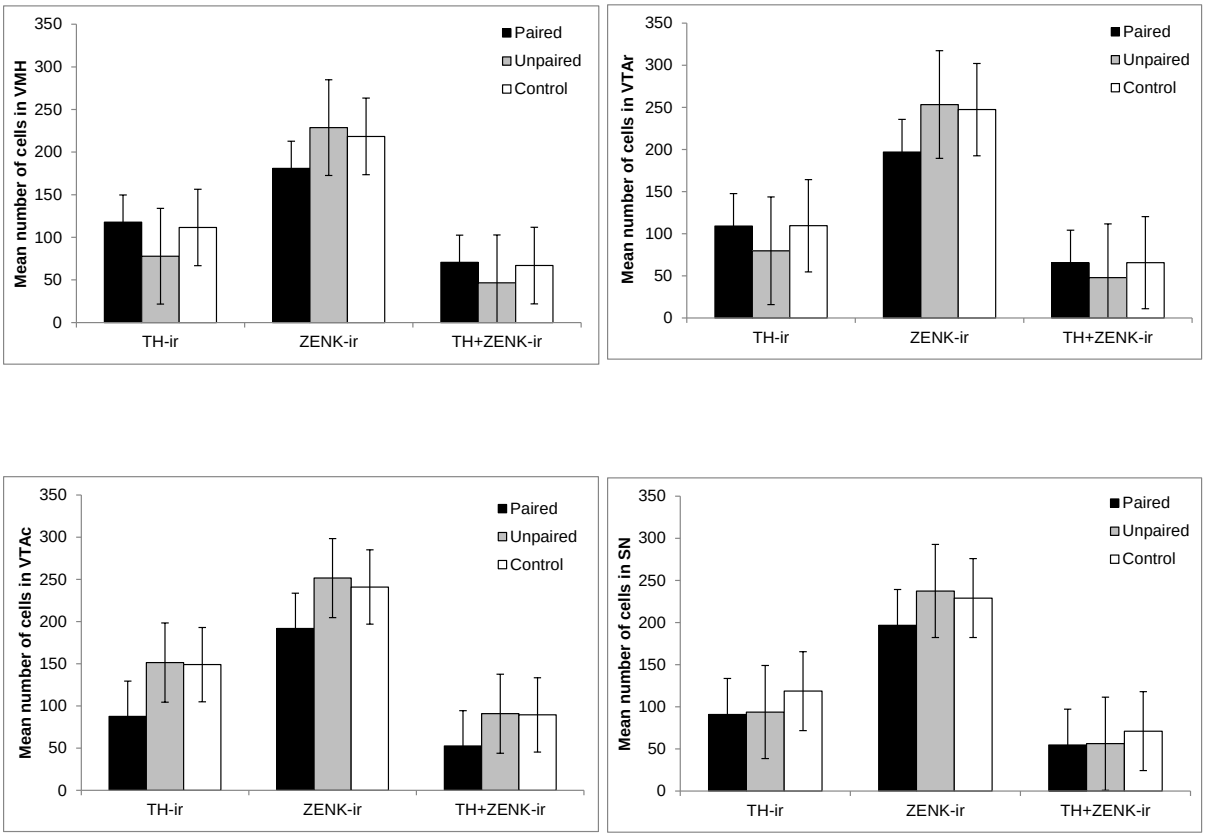


Figure 3.5. Examples of TH-ir, ZENK-ir, and TH+ZENK-ir in NCM, CMM, and VMH of adult female zebra finches paired for 48hr, unpaired, or control. 400X magnification.

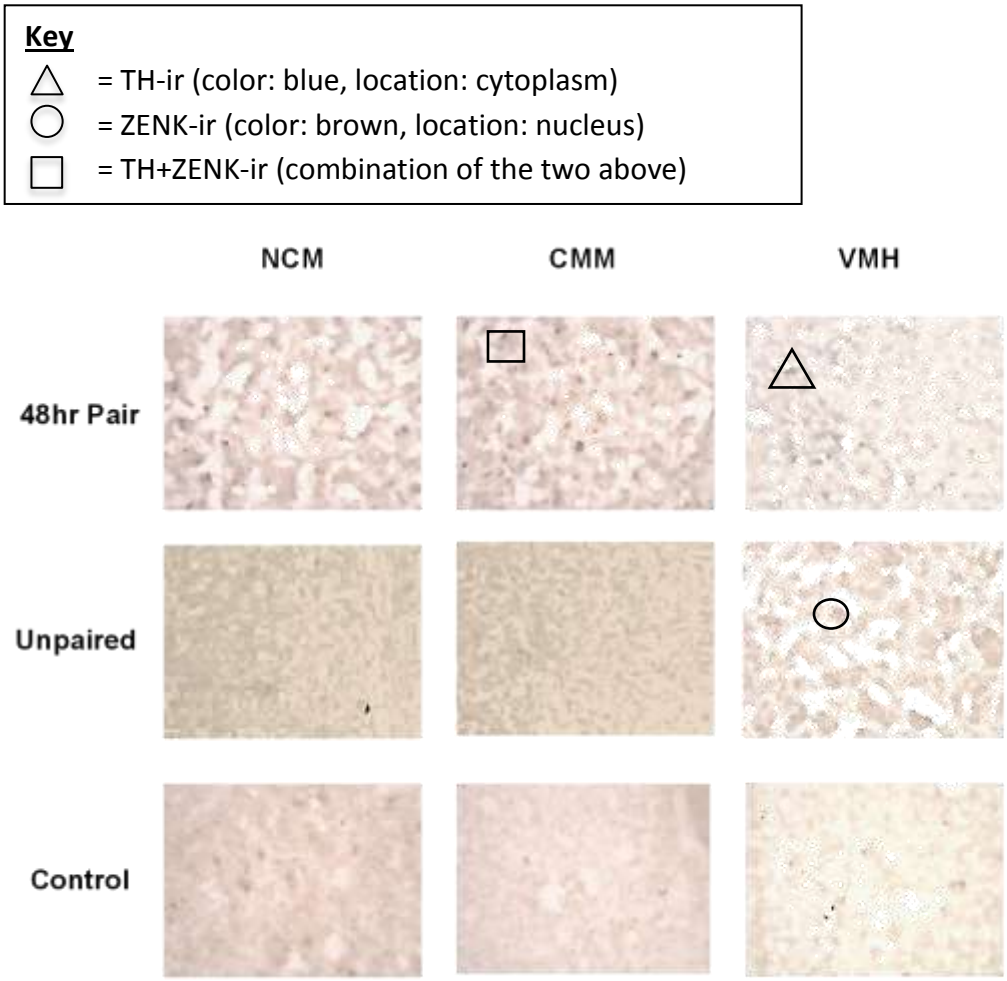
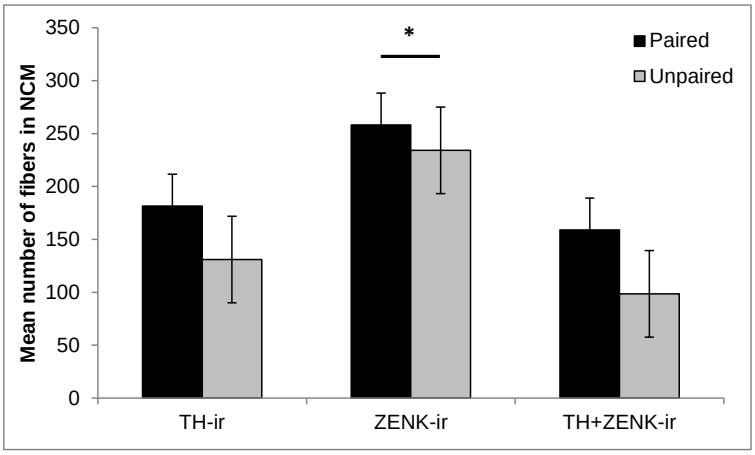
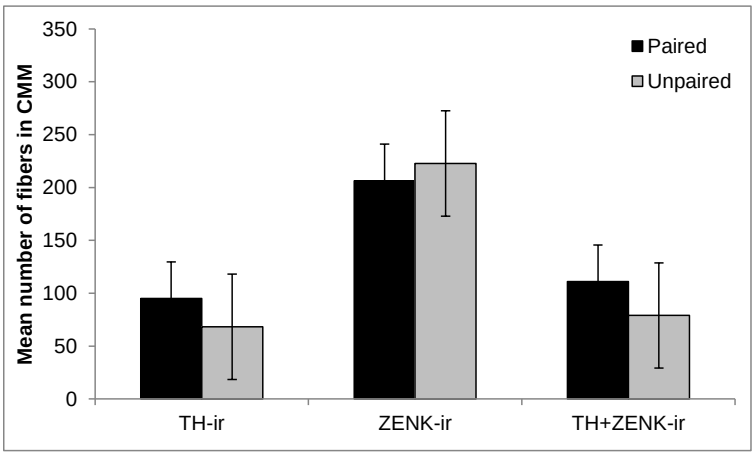


Figure 3.6. Bar graphs represent the mean number of TH-ir, ZENK-ir, and TH+ZENK-ir expressing fibers for NCM, CMM, and VMH in female zebra finches. Statistical significance is indicated by the * and error bars represent the standard error.





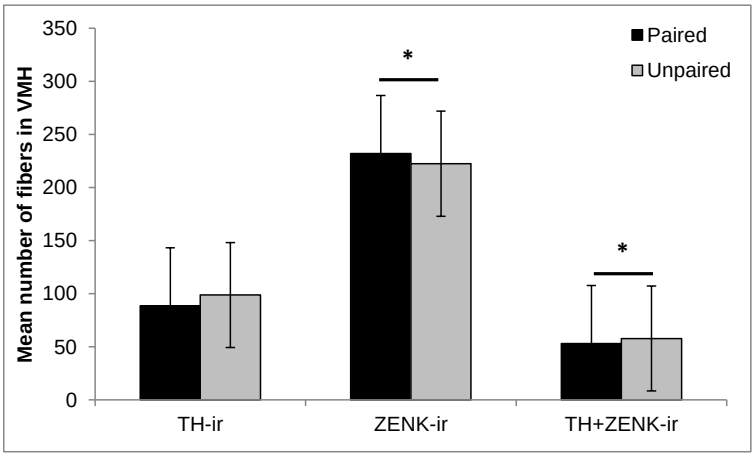


Figure 3.7. Examples of TH-ir, ZENK-ir, and TH+ZENK-ir in VTAr, VTAc, and SNc of adult female zebra finches paired for 48hr, unpaired, or control. 400X magnification.

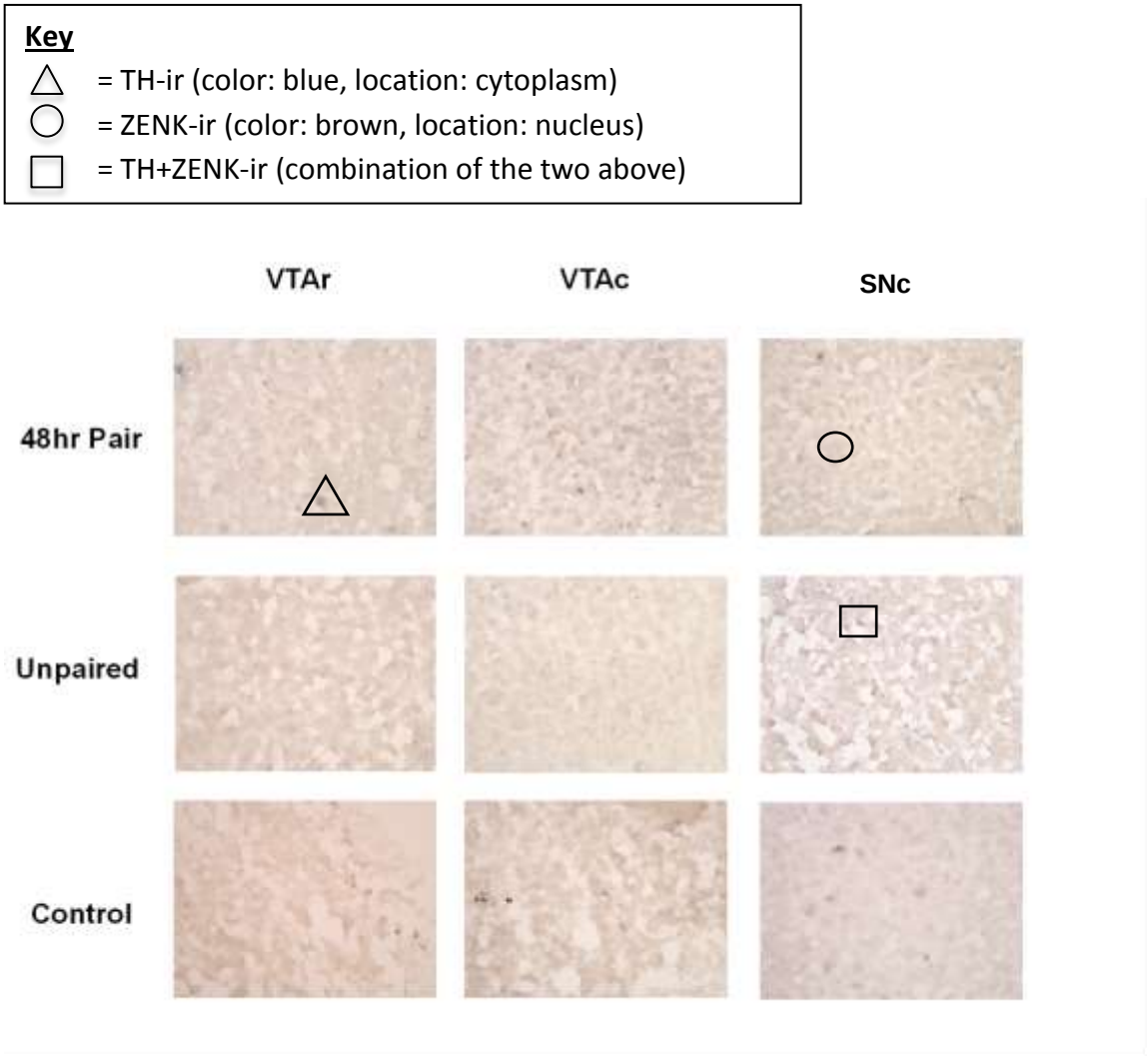
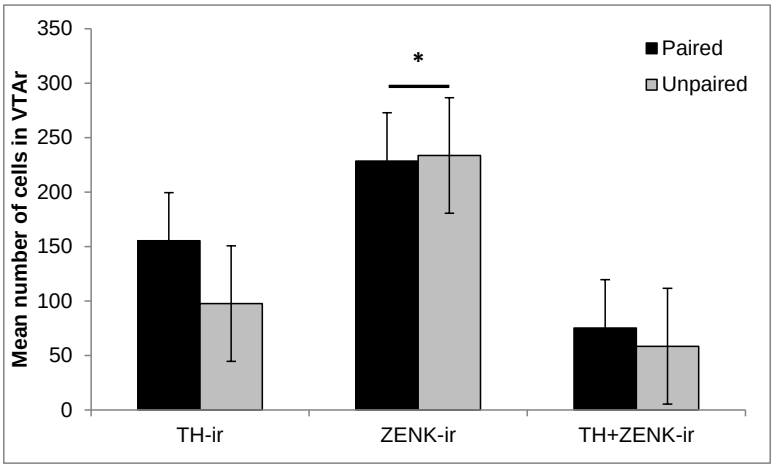
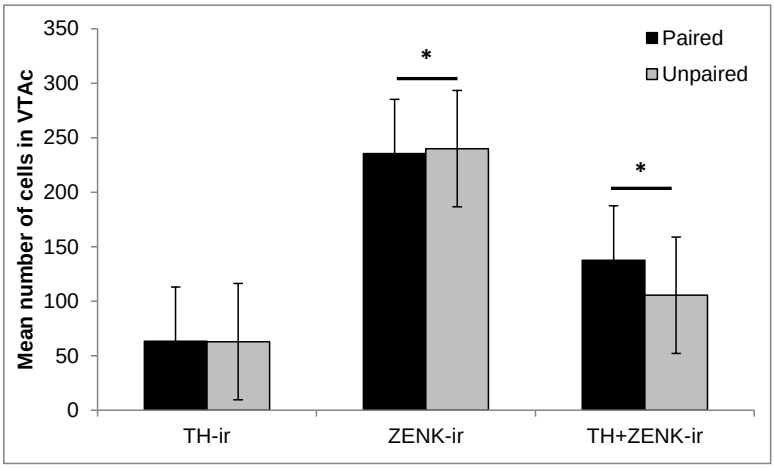
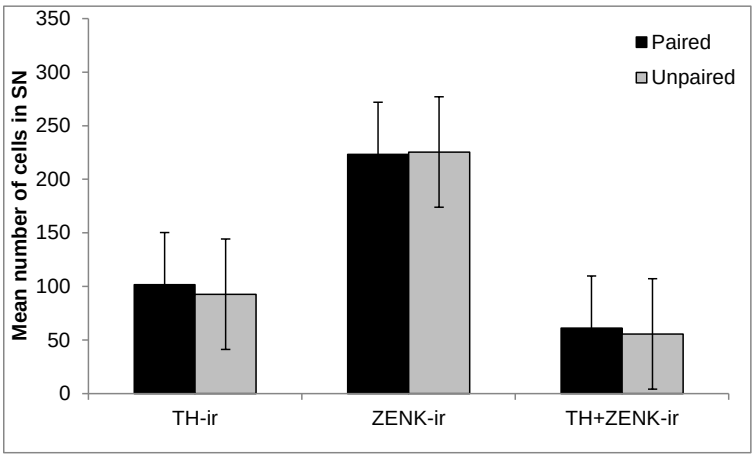


Figure 3.8. Bar graphs represent the mean number of TH-ir, ZENK-ir, and TH+ZENK-ir expressing fibers for VTAr, VTAc, and SNc in female zebra finches. Statistical significance is indicated by the * and error bars represent the standard error.







CHAPTER 4 – THE RELATIONSHIP BETWEEN ACTIVATION OF NORADRENERGIC RECEPTORS AND COURTSHIP AND PAIRING BEHAVIORS IN MALE AND FEMALE ZEBRA FINCHES

Despite the fact that DA seems a likely mediator in the courtship and pairing behaviors of male and female zebra finches, our results were conflicting. TH+ZENK-ir co-expression was only found in Area X of males and the VMH and VTAc of females, and the relationship to pairing was inconsistent. It is possible that catecholamines mediate courtship and pairing behaviors in the zebra finch via the noradrenergic system.

In songbirds, such as the zebra finch, norepinephrine (NE) plays a critical role in the song learning and production in males (Barclay, Harding et al. 1996; Castelino and Ball 2005; Cornil, Castelino, & Ball, 2008; Heimovics et al., 2011; Riters & Ball, 2002) and song perception in females (Appeltants, Del Negro et al. 2002; Sockman & Salvante, 2008; Vyas, Harding et al. 2008; Lynch, Diekamp et al. 2012). Furthermore, the brain areas underlying the song system have been implicated in learning and memory (Bolhuis, Zijlstra et al. 2000; Bolhuis, Hetebrij et al. 2001; Terpstra, Bolhuis et al. 2004; Bolhuis and Gahr 2006). Therefore, studying NE in the zebra finch is a useful mechanism for understanding the cognition underlying learning and memory.

NE has been implicated in social recognition (Dluzen, Muraoka, & Landgraf, 1998) and social memory (Griffin & Taylor, 1995) in the rat, an important part of social relationships. Social recognition in the zebra finch is accomplished through individual song and NE is thought to play a role in the production and learning of song in males (Castelino & Ball 2005; Cornil, Castelino et al., 2008; Riters & Ball, 2002) and in the perception and memory of song in females

(Appeltants, Ball, & Balthazart, 2001; Lynch, Diekamp et al., 2012; Velho, Lu, et al., 2012). Whereas the role of NE in the avian song system has been established, less is understood of how NE is involved in the formation of social relationships. Thus, studying the neural substrates of song production and perception in zebra finches can provide insights into human social behavior, as few animal taxa display vocal learning. The similarities between the imitation process between humans and songbirds (i.e. learning from a parent or "tutor") make the zebra finch a unique animal model for studying vocal communication. The zebra finch (*Taeniopygia guttata*) is a highly gregarious avian species that lives in large social groups, forms lifelong monogamous pair bonds, and uses visual and vocal cues for social recognition (Zann, 1996), much like humans. Given these similarities, the zebra finch is an excellent model for studying the role of NE in the formation of social relationships at a molecular level.

NE has been well studied in the zebra finch song and auditory systems (Barclay, Harding et al. 1996; Mello, Pinaud et al. 1998; Newman 1999; Riters and Ball 2002; Cornil, Castelino et al. 2008; Velho, Lu et al. 2012). NE is an exciting candidate mechanism for studying the formation of social relationships in zebra finches given the prevalence of NE in the brain - which contains 10 times the amount of NE compared to mammals (Barclay and Harding 1988; Waterman and Harding 2008). Furthermore, our understanding of the neurobiology of the song system and genome are well established (Nottebohm, Alvarez-Buylla et al. 1990; Nottebohm 1991; Nottebohm 2002; Reiner, Perkel et al. 2004) thus, NE deserves further study in the formation of social relationships in the zebra finch.

High densities of catecholaminergic innervations have been documented within the song system through immunocytochemical studies using dopamine-beta-hydroxylase (DBH; Bottjer,

1993; Soha, Shimizu, & Doupe, 1995; Appletants, Ball, & Balthazart, 2001; Mello, Pinaud, & Ribeiro, 1998; Castelino, Diekamp, & Ball, 2007; Lynch, Diekamp, Ball 2008). The alpha 2c adrenergic receptor (ADRA2c) has been found predominantly in song and motivation brain areas in male zebra finches (Riters & Ball 2002; Cornil, Castelino et al. 2008; Heimovics et al 2011). Particularly high levels of ADRA2c have been found in the song regions HVC, RA and Area X of males (Ball & Balthazart, 2007; Cornil, Castelino et al. 2008; Riters and Ball 2002).

Further evidence of noradrenergic action in the song system comes from research in which noradrenergic fibers are destroyed using a specific noradrenergic neurotoxin, DSP-4. Following injection of DSP-4, ZENK-ir increases in Area X as a result of directed singing (Castelino and Ball 2005). ZENK is low when males are engaged in directed, or courtship singing, but high during undirected, or non-courtship, song (Jarvis et al., 1998, Kimpo & Doupe, 1997). Therefore, DSP-4 administration modifies the motivational aspects of directed song in Area X (Castelino and Ball 2005), although the mechanism by which this occurs is unclear.

NE is implicated in male song production and it may also be critical to song perception in females, both of which affect pairing behavior. Increases in the co-localization of TH and ZENK cells in the locus coeruleus (LoC) occur in song-exposed female zebra finches (Lynch, Diekamp et al. 2012), indicating activation of NE in response to hearing courtship song from a male. Depletion of NE decreases female mate choice in songbirds (Vyas, Harding et al. 2008). Females treated with a NE neurotoxin (DSP-4) do not discriminate between control males and DSP-4 males, whereas control females show a significant preference for control males over DSP-4 males (Vahaba, Lacey, & Tomaszycki, 2013). DSP-4 treatments (Lynch and Ball 2008) and treatments of alpha-adrenergic antagonists both decrease ZENK expression in NCM of females

(Velho, Lu et al. 2012). Also, ZENK-ir is significantly reduced in female canaries treated with DSP-4 in the NCM and CMM (Lynch and Ball 2008). Induction of ZENK is a proposed mechanism of synaptic plasticity and memory consolidation (Jarvis & Nottebohm, 1997; Mello, 2002; Knapska & Kaczmarek, 2004), making it useful in the study of avian song and a mediator of pairing behavior. Discerning high quality male song is critical to mate choice in the female zebra finch (Riebel 2000, 2003; Tomaszycki & Adkins-Regan, 2005) and evidence indicates that NE action in the female auditory system is critical to this process. ADRA1d is the most prevalent alpha adrenergic sub-type in the caudomedial nidopallium (NCM - Velho, Lu et al. 2012), and is therefore a promising avenue for study in females.

The goal of the current study is to understand the role of NE in social relationships in both male and female zebra finches. This will be accomplished by analyzing ADRA2c (in males), ADRA1d (in females), and ZENK in the song system (HVC, RA, LMAN, Area X) and auditory system (NCM and CMM) of paired, unpaired, and control zebra finches.

Method

Subjects

Subjects are the same as those used in Ch.3.

Brain Collection

Brains were collected as in Ch.3.

Probe Preparation

Probes were derived from published sequences (ADRA2c: Genbank:XM_002195081; ADRA1d: Genbank:XM_002187963). We developed primers for both using the NCBI primer tool (ADRA2c: Forward = ACGTGCTGTTCTGTACCTCG; reverse =

GCTACTGGAGTGCTTGGAGG and ADRA1d: Forward = CTGGCCGTCTTCATCCTCTC; reverse = CTGGCCACCACGTAAATCCT, beginning at 345bp) and conducted reverse-transcribed PCR (#12574-035, Invitrogen, Carlsbad, CA) per manufacturer's instructions on isolated RNA to obtain the DNA. The resulting products from both ADRA2c and ADRA1d were sequenced using central facilities (Wayne State University) and sequence identity for zebra finch ADRA2c and ADRA1d was confirmed using the BLASTn tool on the NCBI website. Product size for ADRA2c (611bp) and ADRA1d (539bp) was also confirmed. Both probes were prepared using Roche Applied Science DIG RNA Labeling kits according to manufacturer's instructions (catalog # 11175025910, Indianapolis, IN). Probe concentrations were determined using a dot blot assay (Lowrey & Tomaszycki, 2014).

In Situ Hybridization and Immunocytochemistry (ICC)

Fluorescence *in situ* hybridization and ICC was conducted using a protocol derived from Wu et al. (2010). Slides were fixed with 3% paraformaldehyde, acetylated, dehydrated, and air dried. Hybridization occurred at 55°C overnight with 1:1000 concentration of ADRA2c (for males only) or ADRA1d (for females only) probe in hybridization buffer. After a series of washes, slides were incubated in 0.3% hydrogen peroxide in TNT buffer for 10 minutes, and blocked in TNB Buffer for a half hour. Slides were then incubated in the secondary antibody (1:200, Anti-DIG-POD, #11207733910, Roche Applied Science, Indianapolis, IN) for 2hr, followed by 30 minutes in a tyramide-conjugated fluorophore (1:100, TSA kit#22, Alexa 488, Invitrogen, Carlsbad, CA). Slides were then rinsed twice in TNT buffer and blocked for 30 minutes in 2% normal goat serum and 0.3% Triton X in PBS at room temperature. Slides were incubated for 48 hours in primary ZENK antibody (anti-rabbit IgG 1:1000, Santa Cruz

Biotechnology, Dallas, TX, #SC189) at 4°C. Slides were rinsed three times in PBS and incubated in biotinylated goat anti-rabbit serum (1:250; Vector Laboratories, Burlingame, CA) in 2% NGS, 0.3% Triton X, and tyramide-conjugated fluorophore (1:100, TSA kit#15, Alexa 594, Invitrogen, Carlsbad, CA) in TBS for 2 hours. Slides were then rinsed 3 times in PBS and rinsed once with distilled water. Finally, slides were cover-slipped with Slow Fade (#S36937, Invitrogen, Carlsbad, CA), cured overnight, and sealed with nail polish.

Quantification

Regions were quantified using a Nikon Eclipse 80i microscope and Nikon Elements Software (AR 3.0). The observers (KM, PK, and ELO), who were blind to treatment groups, quantified the number of cells in each brain area. For cells containing ADRA1d (females), ADRA2c (males), ZENK (both sexes) or ADRA/ZENK co-expression (both sexes), 6 brain regions were investigated. These include the song areas Area X, LMAN, HVC, and RA in males and the auditory areas CMM and NCM in females. These regions were located using adjacent slides stained with thionin. On average, 3 sections (representing both left and right hemispheres) were quantified per animal.

Statistical Analysis

All data were analyzed in SPSS v.22 (SPSS Inc. 2013, Chicago, IL). Prior to analysis, the mean number of cells expressing ADRA (2c for males and 1d for females), immunopositive for ZENK, and co-localization of ADRA and ZENK across regions, and measured behaviors were log-transformed to achieve normality. Two control treatment groups were created: birds that remained unpaired despite the opportunity to pair, and those who were housed in same sex aviaries and not allowed to pair. Differences in behavior by treatment and regional expression

were tested in a 3 (cell expression: ADRA, ZENK, co-localization) · 3 (treatment group: Paired, Unpaired, Control) multivariate analysis of variance (MANOVA). Due to the limitations of sample size and multicollinearity of cell expression across regions (all r = 0.49-0.81), separate MANOVAs were run by region depending on sex (Males: Area X, LMAN, HVC and Females: CMM and NCM) Bonferroni corrections where appropriate. Effect sizes (η2) are also reported. Due to an inability to adequately sample tissue, 3 unpaired and 3 control female cases were lost, resulting in an inadequate sample size. To allow for multivariate analyses, the control group in the female birds was defined as both unpaired and same-sex controls (n = 6). Measures of cell expression in RA were insufficient for statistical analyses and therefore the region was omitted from hypothesis testing.

The relationship between pairing and courtship behaviors and ADRA+ZENK expression was analyzed using multiple linear regressions. ADRA, ZENK, and co-expression cell numbers were averaged across each hemisphere in each section and log-transformed. To test the ability of behaviors to predict expression, ADRA, ZENK, and co-expression in brain areas that were statistically significant were entered as dependent variables, and behaviors (directed singing, clumping, nesting, and allopreening) were entered as independent variables in the multiple linear regression. Copulations and tail quivers occurred too infrequently to permit inclusion in the analyses. The multiple linear regressions were run for each sex separately. Stepwise multiple linear regressions were run; only models with the highest adjusted R^2 values are presented, as in earlier work (Alger et al., 2011). All models and associated p-values are reported in Table 1.

Results

Association between Regional Expression and Pairing in Males

ADRA2c , ZENK, and co-expression in male zebra finches. The effect of Pairing was statistically significant for co-expression in Area X, but not for ADRA2c or ZENK-ir expression alone (ADRA2c: $F_{2,15}$ = 3.02, p = 0.09, η^2 = 0.34; ZENK: $F_{2,15}$ =3.28, p = 0.08, η^2 = 0.35; co-expression: $F_{2,15}$ = 5.41, p = 0.02, η^2 = 0.47; see Figure 1). Bonferroni post hoc tests revealed that the paired group (M = 58.0, SE = 11.0) had a higher number of cells co-expressing ADRA2c mRNA + ZENK-ir than the unpaired group (M = 12.4, SE = 9.0; p = 0.02). There was no significant difference between the paired group and the control group (M = 22.0, SE = 9.8; p = 0.09, see Figure 1). There was also no significant difference between the unpaired and control group (p = 1.00, see Figure 1)

The effect of Pairing was statistically significant for co-expression in LMAN, but not for ADRA2c or ZENK-ir expression alone (ADRA2c: $F_{2,15}$ = 2.34, p = 0.14, η^2 = 0.28; ZENK: $F_{2,15}$ = 1.95, p = 0.19, η^2 = 0.25; co-expression: $F_{2,15}$ = 3.83, p = 0.05, η^2 = 0.39; see Figure2). Bonferroni post hoc tests revealed that the paired group (M = 71.8, SE = 13.8) had a higher number of cells co-expressing ADRA2c mRNA + ZENK-ir than the unpaired group (M = 23.8, SE = 11.3), however, this difference was not statistically significant (p = 0.06). There was no significant difference between the paired group and the control group (M = 32.6, SE = 12.4; p = 0.17) or between the unpaired group and the control group (p = 1.00).

The effect of Pairing was not statistically significant for ADRA2c, ZENK-ir, or co-expression in HVC (ADRA2c: $F_{2,15}$ = 0.86, p = 0.45, η^2 = 0.13; ZENK: $F_{2,15}$ = 0.37, p = 0.70, η^2 = 0.06; co-expression: $F_{2,15}$ = 1.31, p = 0.31, η^2 = 0.18; see Figure 3).

Association between Regional Expression and Pairing in Females

ADRA1d , ZENK, and co-expression in female zebra finches. In the NCM, the effect of Pairing was not statistically significant for ADRA1d mRNA, ZENK-ir, or co-expression (ADRA1d: $F_{1,13}$ = 1.29, p = 0.28, η^2 = 0.11; ZENK: $F_{1,13}$ = 2.81, p = 0.12, η^2 = 0.20; co-expression: $F_{1,13}$ = 0.09, p = 0.77, η^2 = 0.01; see Figure 4).

The effect of Pairing was not statistically significant for ADRA1d mRNA, ZENK-ir, or co-expression in CMM (ADRA1d: $F_{2,10}$ = 1.13, p = 0.36, η^2 = 0.18; ZENK: $F_{2,10}$ = 0.84, p = 0.46, η^2 = 0.14; co-expression: $F_{2,15}$ = 0.25, p = 0.78, η^2 = 0.05; see Figure5).

Contributions of Behavior to Variations in ADRA2c+ZENK-ir Co-expression

In Area X of males, directed singing, clumping, nesting, and allopreening behavior predicted variations in the number of cells expressing ADRA2c+ZENK-ir co-expression (see Table 1). However, only nesting and allopreening were statistically significant (see Table 1). Only allopreening significantly predicted the number of cells expressing ADRA2c+ZENK-ir co-expression in LMAN of male zebra finches.

There were no significant effects of pairing on molecular expression in females, so no linear regressions were run for the NCM or CMM.

Discussion

This was the first study to investigate the effects of forming a social relationship on expression of ADRA mRNA in zebra finches. Both ADRA and ZENK have been implicated in brain areas important for learning and memory — functions that are relevant to pairing. ZENK-ir is a protein marker for activation in brain areas important for learning and memory (Mello & Clayton, 1994; Mello, 2002; Knapska & Kaczmarek, 2004) and has been studied in relation to social cues (Goodson, 2005; Heimovics & Riters, 2007; Maney et al., 2003; Robinson, Fernald,

& Clayton, 2008). ADRAs have been implicated in memory processing (Scheinin et al., 1994; Gibbs, Hutchinson, & Hertz, 2008; Hutchinson, Gibbs, & Summers, 2008; Gibbs, Hutchinson, & Summers, 2010) and in the song system (Riters & Ball, 2002; Cornil, Castelino, & Ball, 2008). Here, I demonstrate that an increase in ZENK + ADRA2c in song areas is associated with increases in courtship behaviors by males, and the relationship is stronger in paired birds than in unpaired birds.

ADRA2c + ZENK-ir co-expression increased in song areas of paired male zebra finches.

ADRA2c is proposed to be a neural substrate of singing in male zebra finches. Here, male zebra finches that were in a pair bonded relationship for 48 hours showed higher ADRA2c+ZENK-ir co-expression in song areas Area X and LMAN. Receptor autoradiography studies in the zebra finch have shown high densities of ADRA2 in these same regions (Riters & Ball, 2002; Cornil, Castelino, & Ball, 2008). Evidence for specific action in these areas comes from co-localization of ADRA2c + ZENK.

ZENK-ir is low in Area X when males are engaged in directed singing toward another female or male, but high when singing alone (Jarvis et al., 1998, Kimpo & Doupe, 1997). Male canaries that produce song show ZENK-ir in HVC, RA, Area X, and LMAN and the number of cells immunopositive for ZENK is linearly proportional to the amount of song produced (Jarvis & Nottebohm, 1997). Increased singing in paired male zebra finches resulted in increased levels of ADRA2c + ZENK co-expression in Area X and HVC. This is the first study, to our knowledge, to investigate the effects of courtship on ADRA2c + ZENK co-expression in the male zebra finch. Based on these findings, ADRA2c receptors in Area X and LMAN are active during the pairing process in male zebra finches.

A linear regression showed that nesting and allopreening behavior significantly predicts variations in ADRA2c+ZENK-ir co-expression in Area X of male zebra finches. Given that these are behaviors that are only expressed after the pairbond has been formed; it is possible that ADRA2c plays a role in forming the pairbond. Allopreening also significantly predicts ADRA2c+ZENK-ir co-expression in LMAN. Although singing was more prevalent in paired males and plays a major role in the courtship process, it did not significantly predict variation in ADRA2c+ZENK-ir.

The ADRA2c receptor sub-type has been linked to memory, and this may be the role that it plays in the formation of social relationships. ADRA2c mRNA is localized to the basal ganglia, cerebral cortex and hippocampus in mammals (Scheinin et al., 1994), brain areas involved in memory processing. Activation of ADRA2c increases the synthesis of glycogen in the forebrain and inhibiting glycogen breakdown impairs memory formation (Gibbs, Hutchinson, & Hertz, 2008; Hutchinson, Gibbs, & Summers, 2008). Glycogen that is present in astrocytes plays an important role in memory processing, but it is not known whether or not ADRA2cs are performing this function in the LoC or other brain areas (Gibbs, Hutchinson, & Summers, 2010). ZENK also demonstrates specificity for patterns of cell activity that lead to neuronal change and ultimately to long-term potentiation and plasticity underlying learning and memory (Mello & Clayton, 1994; Mello, 2002; Knapska & Kaczmarek, 2004). Therefore the role of ADRA2c and ZENK may act through memory functions that are necessary for pair bond formation.

Pairing did not significantly alter ADRA1d + ZENK in female zebra finches.

The present study found that levels of ADRA1d mRNA, ZENK-ir, and co-expression in the NCM and CMM were higher in paired females than in the control group, however, these

differences were not significant. However, these tests were likely underpowered, and therefore did not achieve statistical significance. Females have been largely understudied in animal research (McCarthy et al. 2012; Arnold & Lusis 2012), and this is also true of the zebra finch. ADRA1d is the most prevalent alpha adrenergic sub-type in the NCM (Velho, Lu et al. 2012), but little is known about its function. Further study of the function of ADRA1d in the female zebra finch brain is therefore needed.

Conclusions and Future Directions

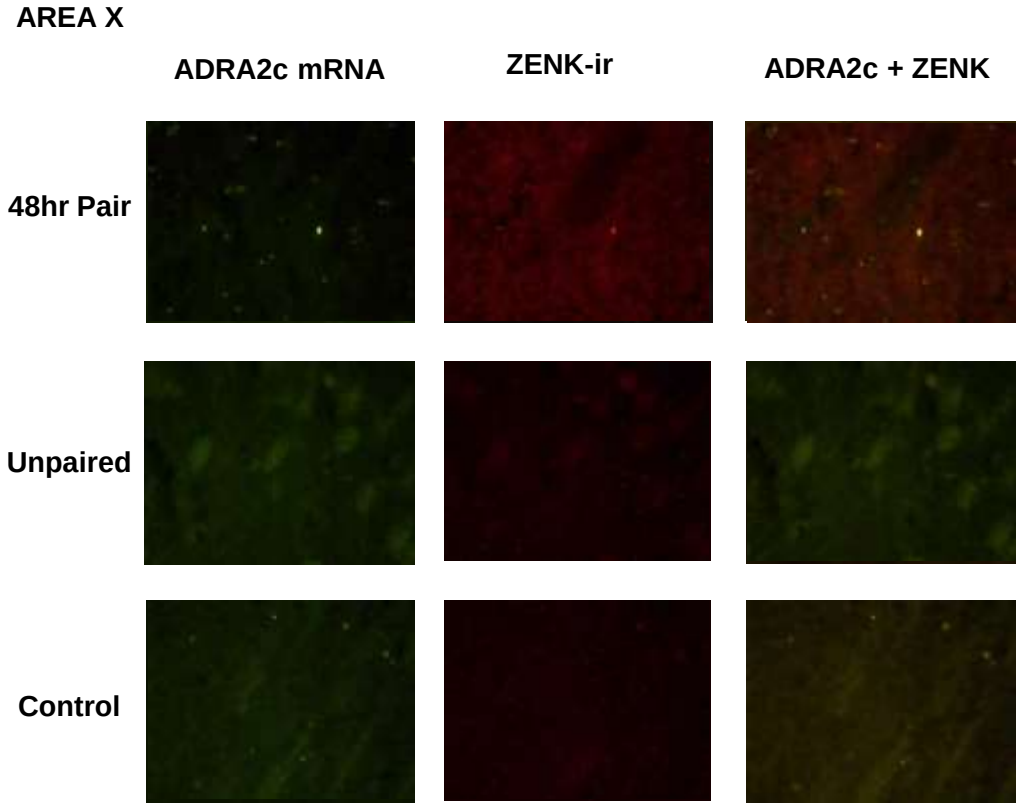
The present study found that paired male zebra finches had higher levels of ADRA2c+ZENK-ir co-expression in song areas Area X and LMAN than unpaired males. These results extend previous findings that directed singing activates noradrenergic receptors (Riters & Ball, 2002; Cornil, Castelino, & Ball, 2008; Mello et al., 1998; Barclay & Harding, 1990; Barclay et al., 1991). These results suggest that noradrenergic receptors are active during the pairing process in song and motivation areas in the male zebra finch.

There were more cells exhibiting co-expression of ADRA1d+ZENK in the NCM and CMM of paired females, but this group difference was not statistically significant. These findings indicate that forming a social relationship, such as a pair bond, has a significant effect on the noradrenergic system in the male zebra finch brain. Social relationships rely heavily on social recognition, learning, and memory and NE and ZENK are both important mechanisms for these behaviors. Future studies should seek to understand the specific actions of post-synaptic ADRA2c receptors in the avian brain and their role in learning and memory in the male zebra finch. The exact location and function of the ADRA1d receptor also deserves further study.

Table 4.1. Regression models of courtship and pairing behaviors that explained variations in ADRA2c+ZENK-ir co-expression in the brain areas of male zebra finches.

Model Statistics	Variables in model	Beta	p-Values
Area X			
Adjusted R^2=0.60, $F_{4, 14}$ = 3.72, p = 0.042	Directed Singing	-0.37	0.146
	Clumping	-0.39	0.183
	Nesting	0.56	0.037
	Allopreening	0.72	0.024
LMAN			
Adjusted R^2=0.53, $F_{3, 13}$ = 5.04, p = 0.043	Allopreening	0.53	0.043

Figure 4.1. Examples of ADRA2c mRNA in Area X of adult male zebra finches paired for 48hr, unpaired, or control. 400X magnification. Bar graphs represent the mean number of ADRA2c mRNA, ZENK-ir, and ADRA2c+ZENK cells for Area X of adult female zebra finches. Statistical significance is indicated by the * and error bars represent the standard error.



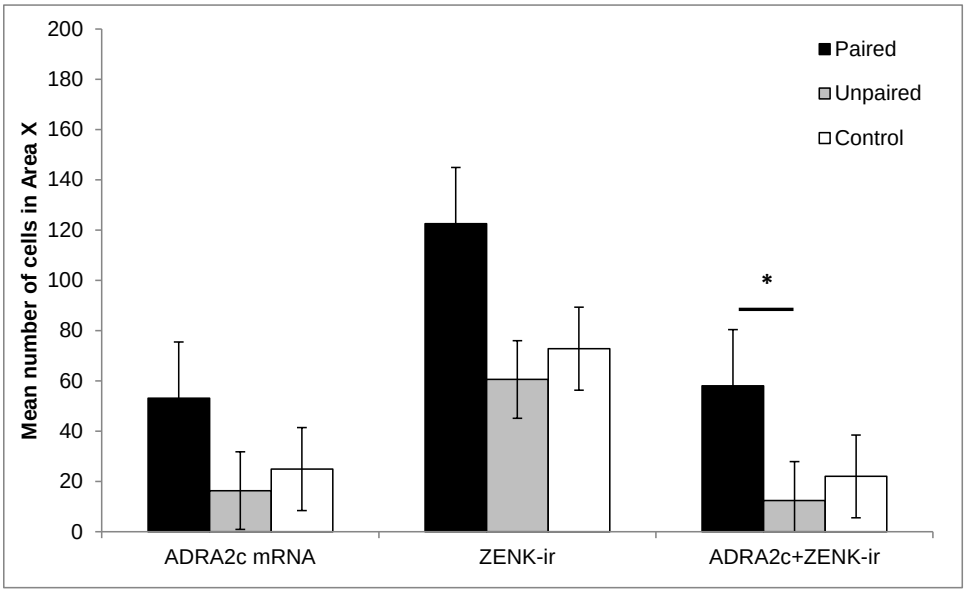
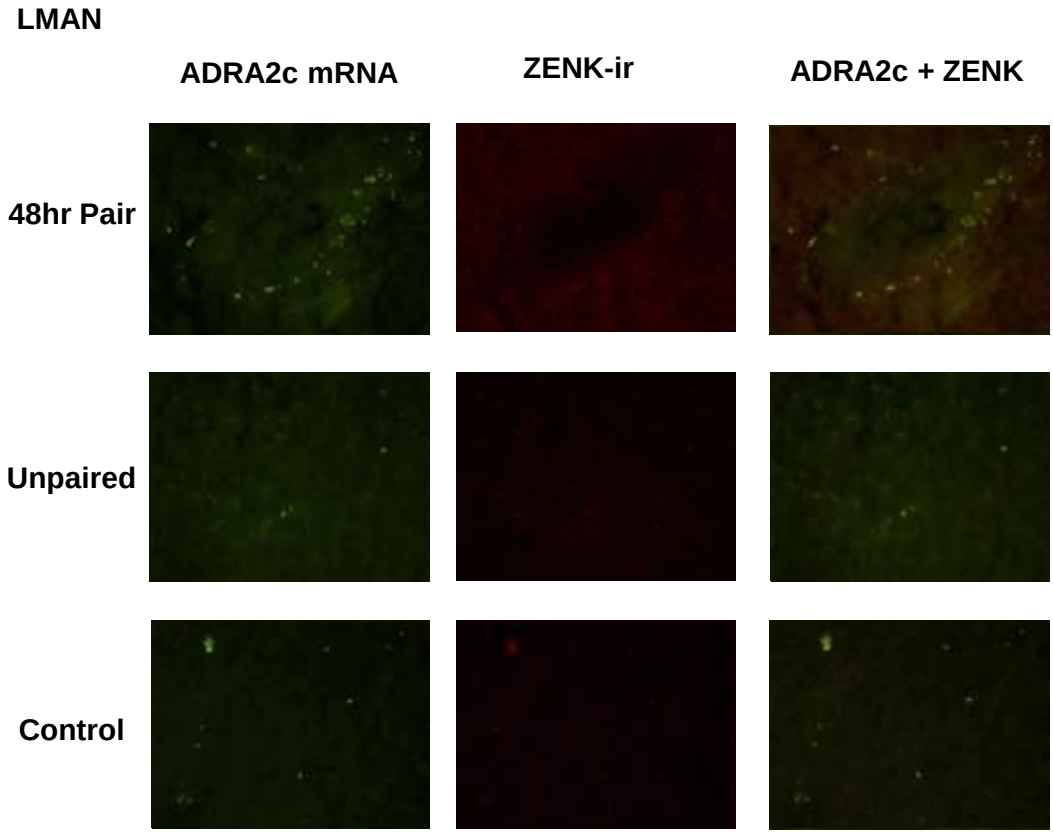


Figure 4.2. Examples of ADRA2c mRNA in LMAN of adult male zebra finches paired for 48hr, unpaired, or control. 400X magnification. Bar graphs represent the mean number of ADRA2c mRNA, ZENK-ir, and ADRA2c+ZENK cells for LMAN of adult female zebra finches. Statistical significance is indicated by the * and error bars represent the standard error.



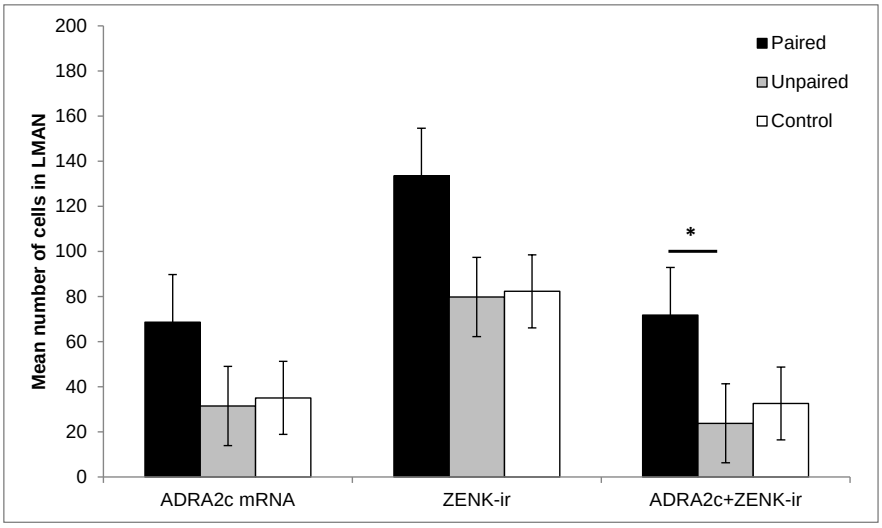
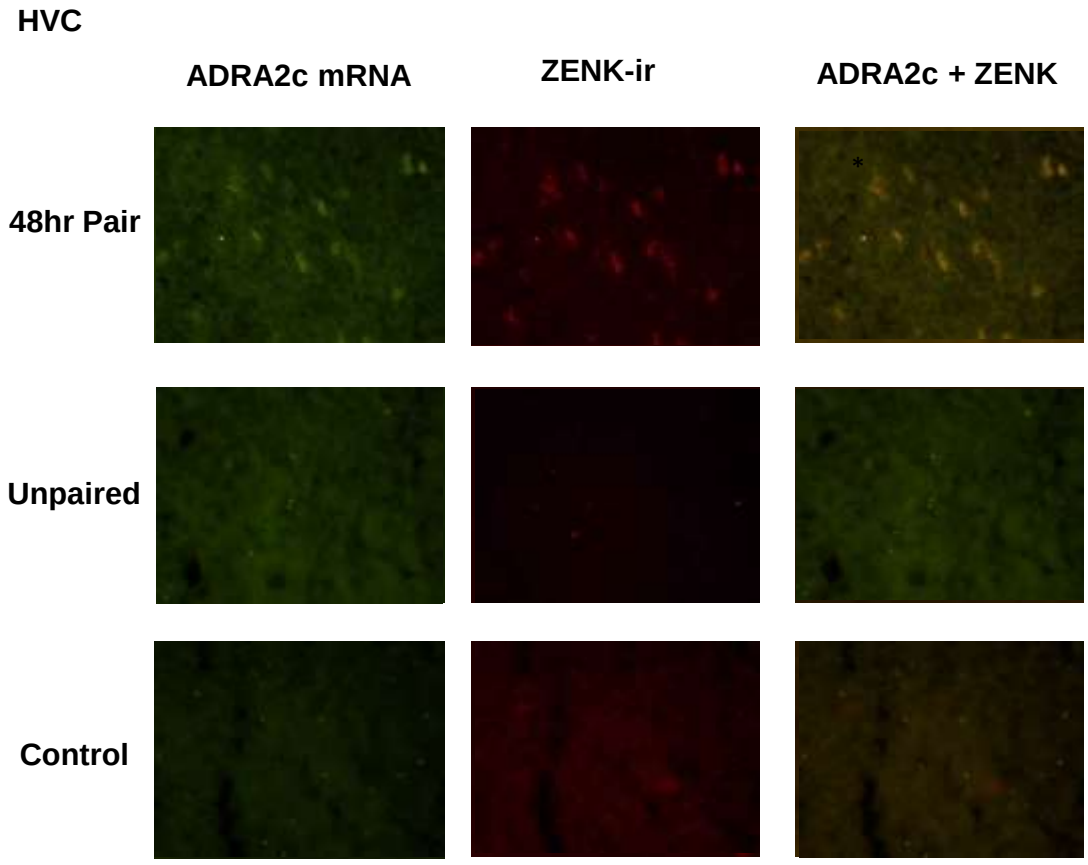


Figure 4.3. Examples of ADRA2c mRNA in HVC of adult male zebra finches paired for 48hr, unpaired, or control. 400X magnification. Bar graphs represent the mean number of ADRA2c mRNA, ZENK-ir, and ADRA2c+ZENK cells for HVC of adult female zebra finches. Statistical significance is indicated by the * and error bars represent the standard error.



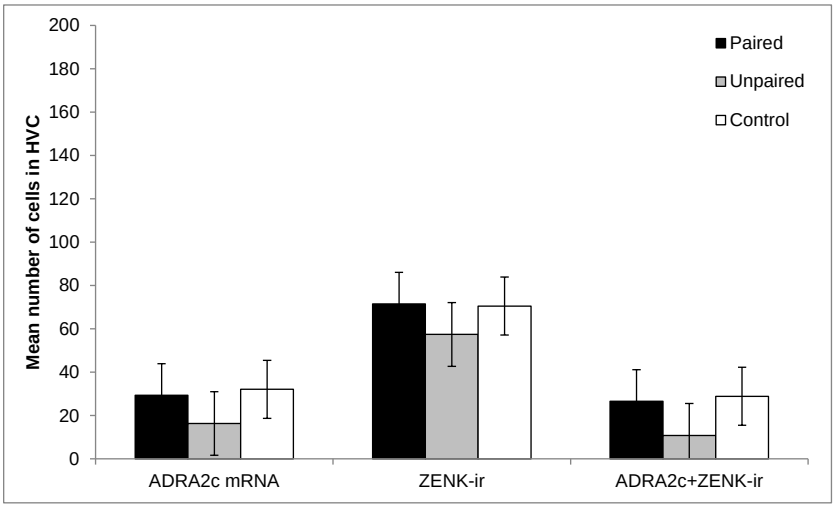
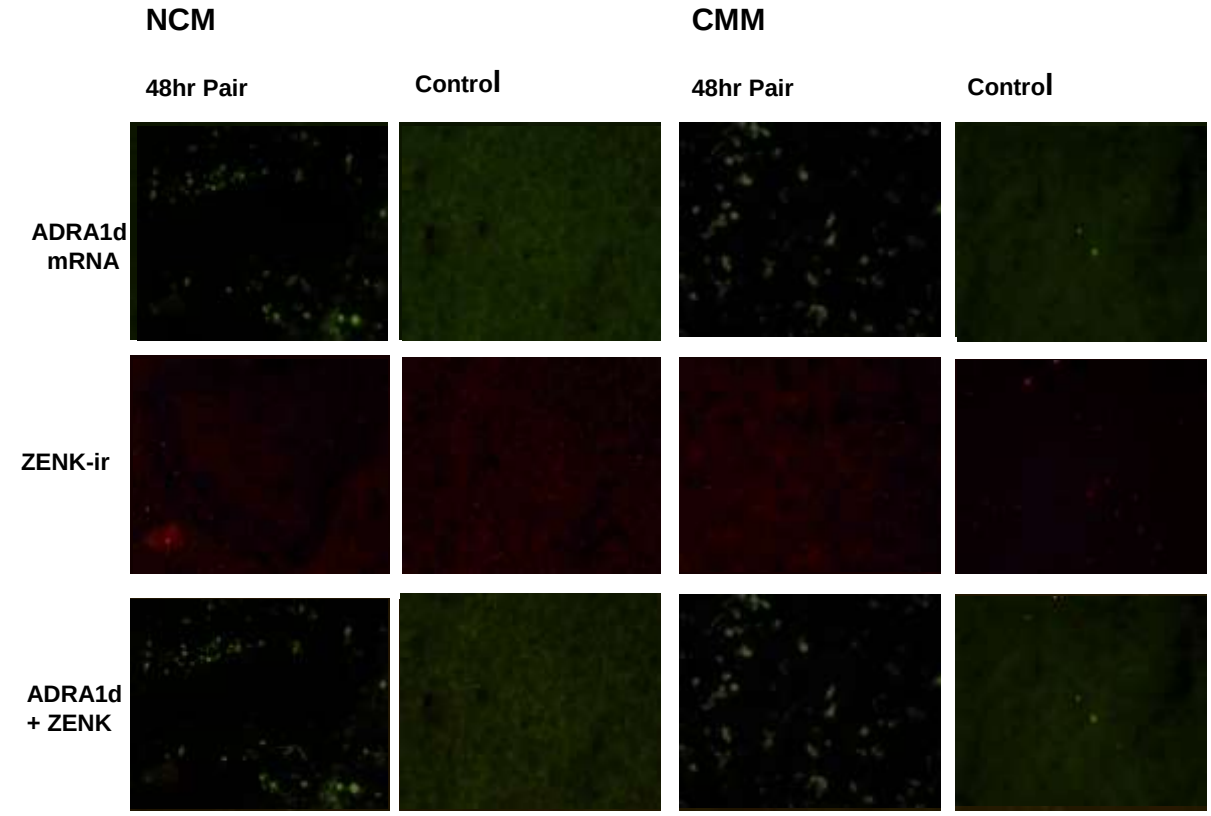
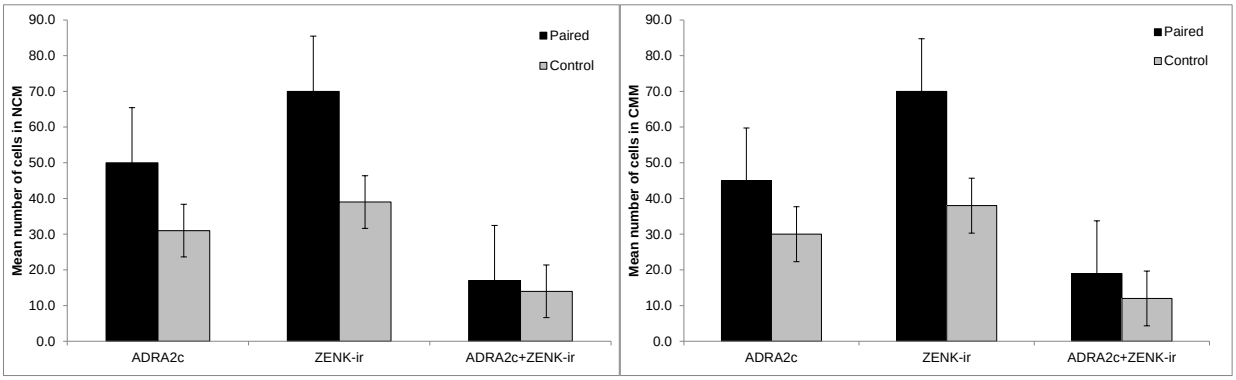


Figure 4.4. Examples of ADRA1d mRNA in NCM and CMM of adult female zebra finches paired for 48hr andcontrol. 400X magnification. Bar graphs represent the mean number of ADRA1d mRNA, ZENK-ir, and ADRA1d+ZENK cells for NCM and CMM of adult female zebra finches. Statistical significance is indicated by the * and error bars represent the standard error.





CHAPTER 5 – THE EFFECT OF AN OXYTOCIN ANTAGONIST ON NORADRENERGIC RECEPTOR MRNA EXPRESSION AND SOCIAL CHOICE IN MALE AND FEMALE ZEBRA FINCHES

The purpose of this collection of studies is to determine if OT modulates the noradrenergic system by increasing the level of ADRA mRNA in the zebra finch. A similar effect has been reported in rodents in which subchronic treatment with OT increased the responsiveness of ADRA2 to an alpha2 agonist in the hypothalamus, amygdala, paraventricular thalamic nucleus (Diaz-Cabiale et al. 2000), and the locus coeruleus (LoC; Petersson et al., 1998). The purpose of the current study is to test the behavioral and molecular effects of blocking OT on ZENK, ADRA2c in male and ADRA1d in female zebra finches. In our first study, we found that both MT and VT mRNA were increased in paired birds as compared to unpaired birds of both sexes. Our results from TH-ZENK-ir studies were inconsistent, but we did find evidence that ADRA2c +ZENK is more prevalent in paired males. Based on previous research and our present findings, we predict that blocking OT will result in increased ADRA2c in song areas of males and will increase ADRA1d in the auditory areas of females.

Social relationships are complex and likely involve multiple neural circuits, including those involved in other cognitive activities, such as learning, memory, motivation, and attention (Bolhuis, Zijlstra et al. 2000; Bolhuis, Hetebrij et al. 2001; Terpstra, Bolhuis et al. 2004; Bolhuis and Gahr 2006). Two neurotransmitters prevalent in these neural circuits are norepinephrine (NE) and oxytocin (OT). NE is associated with learning (Hu, Real et al. 2007), memory (Davis and Squire 1984; Sarter and Markowitsch 1985), motivation (Berridge and Waterhouse 2003), and attention (Aston-Jones and Bloom 1981; Coull, Büchel et al. 1999; Aston-Jones and Cohen

2005). These behaviors are likely important for the formation and maintenance of social relationships. OT regulates affiliative behaviors in animals (Young and Wang 2004; Donaldson and Young 2008; Goodson and Thompson 2010), and is involved in prosocial behaviors such as trust and thinking about a romantic partner in humans (Meyer-Lindenberg, Domes et al. 2011; Churchland and Winkielman 2012). Because these neural substrates overlap with the avian song system (Bolhuis, Zijlstra et al. 2000; Bolhuis, Hetebrij et al. 2001; Terpstra, Bolhuis et al. 2004; Bolhuis and Gahr 2006), it is likely that NE and OT work in concert in the formation and maintenance of social relationships.

NE has been implicated in social recognition (Dluzen, Muraoka, & Landgraf, 1998) and social memory (Griffin & Taylor, 1995), both of which are necessary to form social relationships. NE also plays a role in the production and learning of song in male zebra finches (Castelino & Ball 2005; Cornil, Castelino et al., 2008; Riters & Ball, 2002) and in the perception and memory of song in females (Appeltants, Ball, & Balthazart, 2001; Lynch, Diekamp et al., 2012; Velho, Lu, et al., 2012), which are vital for social recognition and communication. Whereas the role of NE in the avian song system has been established, less is understood about the role of NE in the formation of social relationships. Thus, studying the neural substrates of song production and perception in zebra finches can provide insights into human social behavior, as few animal taxa display vocal learning and rely heavily on vocal communication for social interactions.

Indeed, the few studies of male zebra finch courtship implicate NE in the formation of social relationships. High rates of NE turnover have been found in Area X of males after exposure to a female (Barclay and Harding 1988), implicating this area in motivated courtship

behaviors, such as directed singing. Furthermore, treatment with DSP-4, a noradrenergic neurotoxin, in males increases the latency to sing to females, and results in fewer song bouts and courtship displays (Barclay, Harding et al. 1996) and less directed singing (Vahaba, Lacey, & Tomaszycki, 2013). Overall, DSP-4 treatment increased the latency of males to form a pair relationship, and decreased the likelihood to pair in both sexes (Vahaba, Lacey, & Tomaszycki, 2013).

The alpha-adrenergic receptors (ADRAs) are widely expressed in brain areas controlling song in the male zebra finch. The density of ADRA2 receptors in the song system is higher in males than in females (Riters & Ball 2002). ADRA2s have been localized to song regions: Area X, lateral magnocellular nucleus of the anterior nidopallium (LMAN), HVC (letter based name), and robust nucleus of the arcopallium (RA - Cornil, Castelino, & Ball, 2008; Heimovics et al., 2011; Riters & Ball, 2002). The song system contains two major pathways: the sensorimotor pathway and the anterior forebrain pathway (AFP; (Solis, Brainard et al. 2000). The sensorimotor pathway is necessary for normal song production throughout life and consists mainly of the HVC and RA (Solis, Brainard et al. 2000). The AFP is a basal ganglia-forebrain circuit important in evaluating song feedback and modifying vocal output (Solis, Brainard et al. 2000). The AFP includes mainly Area X and LMAN (Solis, Brainard et al. 2000). The ADRA2c is the most prevalent in the song system and is activated when males engage in directed song (Cornil, Castelino, & Ball, 2008; Heimovics et al., 2011; Riters & Ball, 2002). ZENK is modulated by the noradrenergic system in the song system of male zebra finches (Castelino & Ball, 2005; Cornil, Castelino, & Ball, 2008). ZENK (an acronym of zif-268, egr-1, ngfi-a and krox-24) is an immediate early gene (IEG) that is expressed in areas of the song

system and auditory regions directly in response to singing behavior or song perception (Moorman, Mello et al. 2011).

Female zebra finches do not sing, but choose males on the basis of song (Tomaszycki and Adkins-Regan 2005), so the auditory pathway may be more important for adult females than for adult males. The auditory pathway consists of the caudal medial mesopallium (CMM), caudal medial nidopallium (NCM), and Field L (Jarvis and Nottebohm 1997). The auditory pathway has reciprocal connections to the song system. Exposing zebra finches to conspecific song leads to increased neuronal activation in the NCM and CMM, but not in the song system (Mello, Vicario et al. 1992; Mello and Clayton 1994; Bolhuis, Zijlstra et al. 2000), indicating that these brain areas have a specialized function for auditory processing.

NE is implicated in song perception in females, which affects pairing behavior. Depletion of NE decreases female mate choice in songbirds (Vyas, Harding et al. 2008). Females treated with a NE neurotoxin (DSP-4) do not prefer control males over DSP-4 males, whereas control females show a significant preference for control males over DSP-4 males (Vahaba, Lacey, & Tomaszycki, 2013). DSP-4 treatments (Lynch and Ball 2008) and treatments of alpha-adrenergic antagonists both decrease ZENK expression in NCM of females (Velho, Lu et al. 2012). ZENK-ir is also significantly reduced in female canaries treated with DSP-4 in the NCM and CMM (Lynch and Ball 2008). Induction of ZENK is a proposed mechanism of synaptic plasticity and memory consolidation (Jarvis & Nottebohm, 1997; Mello, 2002; Knapska & Kaczmarek, 2004), making it useful in the study of avian song and a mediator of pairing behavior. Choosing high quality male song is critical for mate choice in the female zebra finch

(Riebel 2000, 2003; Tomaszycki & Adkins-Regan, 2005) and evidence indicates that NE action in the female auditory system is critical to this process. ADRA1d is the most prevalent receptor in the NCM and is more prevalent in song-responsive cells in the NCM than other noradrenergic receptor types (Velho et al. 2012), making it a promising avenue for study in females.

The zebra finch is an excellent model of social relationships. The brain areas involved in song production and audition in birds are analogous to those in the human auditory system (Bolhuis, Okanova, & Scharff, 2010; Doupe & Kuhl, 1999; Jarvis, 2007; Moorman et al., 2012), making zebra finches an ideal translational model for human language acquisition and communication, including learning and memory. The zebra finch is a monogamous species that lives in large social groups, much like humans. In this model, the neurobiology of the song system is well understood, and the genome is fully sequenced and well-annotated — making zebra finches an excellent model for testing the interaction between neural substrates and social relationships.

There is extensive research implicating a role for OT in trust, sociality, parental care, and romantic relationships in mammals (Young and Wang 2004, Donaldson and Young 2008, Goodson and Thompson 2010; Feldman, 2012; Bosch and Neumann 2012; Insel, 2010). In the prairie vole, a rodent model of monogamy, exogenous administration of OT at high doses facilitates pair bonding in both sexes (Cho, DeVries et al. 1999). OT receptors in the nucleus accumbens (NAc) are necessary for pair bonding in female prairie voles (Young, Lim et al. 2001, Liu and Wang 2003) and exogenous administration of OT promotes pair bonding in the absence of mating (Liu and Wang 2003). Affiliative behaviors between members of a monogamous pair

account for natural variations in urinary OT in both sexes of cotton-top tamarins (Snowdon, Pieper et al. 2010). In humans, OT levels positively correlate with more interactions with a romantic partner (see Bartz and Hollander 2006; Feldman 2012; Guastella and MacLeod 2012; Yamasue, Yee et al. 2012 for reviews). Furthermore, OT treatment has been shown to restore social recognition in OT knockout mice (Ferguson, Aldag, Insel, & Young, 2001). Thus, OT appears to affect social relationships in mammals.

There is further evidence from avian models for a role of OT in social relationships. In avian species, species-typical group sizes correlate with mesotocin (MT – the avian homologue of OT) receptor distributions in the lateral septum (LS) regardless of sex (Goodson, Schrock et al. 2009). OT antagonist (OTA) treated zebra finches of both sexes demonstrate an increased latency to pair (Pedersen and Tomaszycki 2012). Paired zebra finches of either sex have higher levels of MT mRNA in the paraventricular nucleus (PVN – location of OT synthesis) than unpaired birds (Lowrey & Tomaszycki, 2014). Furthermore, OTA treated birds that do pair are less likely to remain paired (Klatt and Goodson 2013). Taken together, these studies support the role of OT in social relationships.

Intriguingly, OT and NE are expressed in many of the same brain regions. These brain regions underlie the functions of learning, memory, and motivation functions (Goodson, 2004; Korf, 1981; Mello, Pinaud, & Ribero, 1998; Castelino, Diekamp, & Ball, 2007, 2008; Bottjer, 1993; Soha, Shimizu, & Doupe, 1995). Yet is unclear how OT may interact with NE to constitute either a cohesive mechanism, or an alternate complimentary action, during the formation and maintenance of pairbonds. Previous studies on the relationship between OT and NE has been largely done in the rat, which is a non-monogamous species (Petersson et al., 1998;

Diaz-Cabiale et al. 2000), limiting the validity of conclusions when extended to human relationships. Thus, the present study was designed to address these limitations and to expand our current understanding of the neural basis of monogamous social behavior. Furthermore, we employed a method to quantify ZENK expression as a marker of behavior-specific expression of NE.

Method

Subjects

Subjects were adult wild-type zebra finches (*Taeniopygia guttata*) raised in social aviaries. Subjects were randomly chosen from same-sex cages and randomly assigned to the OTA treated (male n = 6 and female n = 6) and saline treated (male n = 6 and female n = 6) groups. All subjects, regardless of treatment group, were separated from their parents at day 50 post-hatching and housed in same-sex aviaries until the start of the experiment. All subjects were in good health and had not previously formed a pair relationship. Animals were maintained on a timed 12:12 hr light-dark cycle in a temperature (24°C) and humidity (50%) controlled room. Seed and water were provided *ad libitum.* Animals were supplemented with hard-boiled chicken egg, calcium-enriched grain (Simple System Breeder Crumb 5-Day Product, The Bird Care Company) and fresh chopped greens and fruit once per week.

Housing

During tests, subjects were housed in observation cages that were 91.4 ×76.2 × 76.2 cm. Each cage contained a water dish, food dish, grit box, perches, four empty nest boxes, and nesting materials.

OTA Treatment

Each subject in the treatment group was given a 0.05 ml intramuscular injection of 5 µg OTA ([d(CH2)5 1, Tyr(Me)2, Thr4, Orn8, des-Gly-NH2 9]-Vasotocin trifluoroacetate salt, Bachem) dissolved in 0.9% saline, which is an accepted administration method (Goodson et al., 2009; Pederson & Tomaszycki, 2012). The 5 µg dose was chosen because it was the dose that caused the largest effect in our earlier study of pairbonding in the zebra finch (Pederson & Tomaszycki, 2012). The time course for this OTA is unknown. Control animals received 0.05 ml of 0.9% saline.

Behavioral Testing

OT administration has been shown to increase preferences for larger social groups in female zebra finches (Goodson et al. 2009). Blocking OT has been shown to decrease pairing behavior in both sexes (Pederson & Tomaszycki, 2012; Klatt & Goodson, 2013). Therefore, we decided to test the behavioral effects of OTA administration in a two choice test paradigm (see Figure 1). Based on previous research, we hypothesized that blocking OT would eliminate the preference for the opposite sex, as well as for the larger social group.

The test animals were housed together in a separate cage and were kept in the testing room to minimize stress. On the first day of testing, the stimulus animals were selected and placed into one of the two-choice condition test cages (Single Test: 1 Male vs. 1 Female and Group Test: 4 Males vs. 1 Female, see Figure 1) and the test subject was placed in the middle cage. Each test subject was injected and then placed in the testing cage for 15 minutes to allow for habituation and for the drug to take effect. Barriers were placed separating the test cages from the stimulus cages. After the 15-minute habituation period, the barriers were removed and the subject was tested for 10 minutes. After the testing period, the subject was immediately placed

into the second testing condition, with an additional 15 minute waiting period to habituate to the testing cage. The subject was then tested for another 10 minutes. The order of the tests was random and was reversed on the second day of testing (i.e. if a subject received the Single Test first on day 1, they would receive the Group Test first on day 2). In each test, preference was measured by the amount of time (measured in seconds using a timer) the subject spent in the 10cm closest to either social situation (as indicated on the perch using a permanent marker). On the second day of testing the same process was repeated and on the third day, the subjects were injected and euthanized after 1 hr.

Brain Collection

Please reference Ch.4 for details

Probe Preparation

Please reference Ch.4 for details

Dot Blot Assay

Please reference Ch.4 for details

In Situ Hybridization and Immunocytochemistry (ICC)

Please reference Ch.4 for details

Quantification

Please reference Ch.4 for details.

Data Conditioning and Statistical Analysis

All data were analyzed in SPSS v.22 (SPSS Inc. 2013, Chicago, IL). Prior to analysis, the mean number of cells expressing ADRA (2c for males and 1d for females), ZENK, and co-localization of ADRA and ZENK across regions were log-transformed to address non-normality.

Differences in behavior by treatment were tested in a 4 (Condition: Proportion of time spent near a single same-sex bird in the Single Test, Proportion of time spent near the opposite-sex bird in the Single Test 1, Proportion of time spent near the same-sex group in the Group Test, Proportion of time spent near opposite-sex bird in the Group Test) x 2 (treatment group: OTA, Saline) x 2 (sex: Male, Female) multivariate analysis of variance (MANOVA). Significant differences between conditions were followed up with independent t-tests. Differences in behavior by treatment were tested in a 3 (cell expression: ADRA, ZENK, co-localization) x 2 (treatment group: OTA, Saline). The 3 x 2 MANOVA was repeated for each brain region separately to avoid multi-collinearity. Due to the sexual dimorphism of brain regions, these analyses were necessarily conducted for males and females separately.

Results

Two-choice Behavioral Results

The first analysis of differences in behavior by condition, treatment, and sex were conducted using a 4 x 2 x 2 MANOVA. There was no significant effect of OTA treatment ($F_{3, 12}$ = 0.63, p = 0.612), Day ($F_{3, 12}$ = 2.65, p = 0.096), or between sexes ($F_{3, 12}$ = 1.44, p = 0.240) on condition. Therefore, these variables were not included in post-hoc analyses.

Overall, there was a significant main effect of experimental condition. During the Group Test, subjects demonstrated a preference for an opposite-sex bird (percentage of time: M = 31.2%, SE = 0.03) as compared to a group of same-sex birds (percentage of time: M = 13.3%, SE = 0.03): t (15) = -4.02, $p \leq 0.001$. This effect may be partially explained by a subject's preference for a single bird over a group, as there was greater preference for a single opposite-sex bird in the Single Test as compared to the group of same-sex birds in the group Test (t(15) =

-4.10, $p \leq 0.001$). However, the time spent in proximity to the single same-sex bird in the Single Test and group of same-sex birds in the Group Test was similar ($t(15) = 1.98$, $p = 0.07$). Furthermore, when choosing between a single opposite-sex bird and same-sex bird (M = 7.98%, SE = 0.02) in the Single Test, subjects had a preference for the opposite-sex ($t(15) = 6.05$, $p \leq 0.001$). The preference for the opposite-sex bird was equivalent between tests ($t(15) = -0.35$, $p = 0.73$).

Effect of OTA Treatment on ADRA2c mRNA, ZENK-ir, and co-expression in the Male Zebra Finch

OTA treatment significantly lowered ADRA2c mRNA in the anterior forebrain pathway (AFP), which contains Area X and LMAN. There was a significant effect of OTA treatment on ADRA2c mRNA in Area X ($F_{1,16} = 11.49$, $p = 0.004$). Males treated with OTA had significantly fewer cells expressing ADRA2c mRNA than males treated with saline (see Figure 2). OTA treatment had no significant effect on ZENK-ir ($F_{1,16} = 1.04$, $p = 0.32$) or co-expression ($F_{1,16} = 0.01$, $p = 0.94$) in Area X. The effect of OTA treatment on ADRA2c mRNA in LMAN was also statistically significant after correction for multiple comparisons ($F_{1,16} = 6.93$, $p = 0.02$, see Figure 2). OTA treatment had no significant effect on ZENK-ir ($F_{1,16} = 0.24$, $p = 0.63$) or co-expression ($F_{1,16} = 0.19$, $p = 0.67$) in LMAN.

In the sensorimotor pathway, including HVC and RA, there was no significant effect of OTA treatment. In the HVC of male zebra finches, OTA treatment had no significant effect on ADRA2c mRNA ($F_{1,16} = 0.73$, $p = 0.41$), ZENK-ir ($F_{1,16} = 0.001$, $p = 0.97$), or co-expression ($F_{1,16} = 0.008$, $p = 0.93$, see Figure 3). This was also true in the RA: ADRA2c mRNA ($F_{1,16} =$

0.04, p = 0.85), ZENK-ir ($F_{1,16}$ = 0.42, p = 0.53), and co-expression ($F_{1,16}$ = 0.01, p = 0.92) were not significant (see Figure 3).

Effect of OTA Treatment on ADRA1d mRNA, ZENK-ir, and co-expression in the Female Zebra Finch

Female zebra finches treated with OTA showed a significant effect of OTA treatment on ADRA1d mRNA in the NCM ($F_{1,16}$ = 6.88, p = 0.02), such that OTA treated females had fewer cells expressing ADRA1d than did saline treated females (see Figure 4). There was a similar finding in the CMM ($F_{1,16}$ = 5.32, p = 0.04, see Figure 4). OTA treatment had no significant effect on ZENK-ir ($F_{1,16}$ = 0.01, p = 0.95) or co-expression ($F_{1,16}$ = 0.35, p = 0.56) in NCM or in the CMM (ZENK-ir: $F_{1,16}$ = 0.90, p = 0.36, co-expression: $F_{1,16}$ = 4.55, p = 0.06,see Figure 4).

Discussion

Behavioral Effects of OTA Treatment

The two-choice mechanism used for this study was based on a similar design used in Goodson, Schrock, et al. (2009) to assess the effects of OT on social flocking behavior in zebra finches. Their paradigm gave the subject a choice between two or ten same sex conspecifics. Previous work from our lab has shown that administration of an OTA increased latency to pair in both sexes, indicating that OT could affect partner preference. To test this behavioral effect of OT, we used the two-choice paradigm in two contexts. To test for partner preference, subjects were given a choice between an opposite sex bird or a same sex bird. In the second paradigm, the subject was given a choice between an opposite sex bird and 4 same sex birds (see Figure 1 for a visual depiction of the experimental design).

Across treatment groups, birds consistently showed a preference for the opposite-sex in the two-choice tests. Regardless of the comparison to a single same-sex bird or a group of same-sex birds, subjects preferred a single opposite-sex bird. Based upon previous reports of OTA treatment increasing latency to pair (Pedersen and Tomaszycki 2012) and decreased likelihood of remaining paired (Klatt and Goodson 2013), we had expected OTA treatment to affect partner preference. This was not supported in the present analyses. It is plausible that OT does not modify preference, but affects courtship display and perception. Specifically, OTA treatment may alter behavior in social relationships, but not sexual preference. It is also possible that the preference for the single opposite sex bird versus the four same sex birds was not the result of a sexual preference, but actually a demonstration of social preference. The OTA may have affected the subjects' preference for larger social groups. Further study with additional experimental manipulations than included here is necessary to test this hypothesis. A two-choice paradigm is artificial and findings from these types of experimental designs do not always translate to semi-naturalistic settings (Tomaszycki & Adkins-Regan, 2005).

Effects of OTA Treatment in Male Zebra Finches

This is the first experiment to demonstrate a relationship between oxytocin and norepinephrine in the zebra finch brain. We hypothesized that MT (the avian homologue of OT) modulates the noradrenergic system by increasing ADRA mRNA. This hypothesis was based on findings in rodents that subchronic treatment with OT increases the responsiveness of ADRA2 to an alpha2 agonist in the PVN (Diaz-Cabiale et al. 2000), and the LoC (Petersson et al., 1998). We find a similar relationship between OT and the noradrenergic system in that OTA administration decreased ADRA mRNA. We have extended the extant literature by replicating

the effect across the sexes and identified effects in specific brain regions of the avian song and auditory systems.

Although the OTA treatment did not affect partner preference in the choice paradigm, it did affect the noradrenergic system. OTA administration significantly lowered ADRA2c mRNA in the anterior forebrain pathway (AFP) of male zebra finches, but not in the sensorimotor pathway. Therefore, the modulatory effect of OT on NE may be specific to the part of male song system that is directly relevant for courtship and social behaviors, as the AFP is important in evaluating song feedback and modifying vocal output (Solis, Brainard et al. 2000). The effects of OTA on ZENK-ir and co-expression were not significant and we cannot determine if the effect on ADRA2c in the song regions was due to song production. Typically, ZENK-ir is low when males are engaged in directed singing toward another female or male, but high during solo song (Jarvis et al., 1998, Kimpo & Doupe, 1997). The null effect here may be an artifact created from the two-choice paradigm design in which a single opposite-sex bird was always present.

A null effect of ZENK-ir and co-expression in these regions may also be explained by alternate pathways by which nonapeptides may modulate the noradrenergic system in the zebra finch brain. OT has been implicated in sociality and the formation of a monogamous pairbond (Goodson, Schrock et al. 2009; Pedersen and Tomaszycki 2012; Klatt and Goodson 2013; Lowrey & Tomaszycki, 2014). The selectivity of the OTA used in the present experiment is unknown, and it is possible that it could be acting at any of the VT receptors (Goodson et al., 2004, 2009; Leung 2009, 2011; Young & Flanagan-Cato, 2012), and we may have a limited measurement of OT action.

Based on our results, an alternate indirect pathway of OT modulating NE is plausible. For example, nonapeptides are released from the PVN (Koob and Bloom 1982). PVN expression may affect song areas indirectly via efferent projections to VTA in avian species (Korf 1984), which is known to connect with Area X, HVC, and RA (Castelino and Ball 2005; Castelino, Diekamp et al. 2007). There may be additional direct interactions in specific regions. Without additional study of VT receptors, the mechanism of interaction between nonapeptide and noradrenergic systems remains unknown.

The indirect interaction between these two systems may occur, in part, through modification of the sensorimotor pathway, including regions HVC and RA. The sensorimotor pathway is necessary for normal song production throughout life and includes the HVC and RA (Solis, Brainard et al. 2000). Yet, OTA administration did not affect ADRA2c in HVC or RA. The cause of the null effect is unclear, but it is plausible that the modulation of NE by OT in the anterior forebrain is a larger effect. The anterior forebrain is a circuit connecting the basal ganglia and forebrain, including Area X and LMAN, and it is important in evaluating song feedback and modifying vocal output (Solis, Brainard et al. 2000). Neurons in these song system nuclei are activated when the bird is singing (Solis, Brainard et al. 2000; Bolhuis and Gahr 2006). However, Area X has been specifically implicated in the motivational aspects of directed song (Barclay and Harding 1988; Castelino and Ball 2005; Alger, Juang et al. 2011). Previous studies of OTA administration support a decrease in motivation to pair and to remain paired (Pedersen and Tomaszycki 2012; Klatt and Goodson 2013), but here there was no effect on partner preference. Therefore, the effect of nonapeptides on social behaviors via the

noradrenergic system may be more specific to motivational aspects, rather than sensorimotor functions in song production or sexual preference.

Effects of OTA Treatment in Female Zebra Finches

OTA treatment resulted in lower levels of ADRA1d mRNA in female zebra finches in both auditory areas, NCM and CMM. In female zebra finches, who do not sing, the evaluation of male directed song is an extremely important factor in the formation of a pair bond (Riebel, 2000; 2003; Zann, 1999; Tomaszycki & Adkins-Regan, 2005). The auditory brain regions NCM and CMM are the neural substrates of this evaluation function. ADRA1d is the most prevalent receptor in the NCM and is in more song-responsive cells in the NCM than other receptor types (Velho et al. 2012). OTR are expressed in the NCM and CMM (Leung et al. 2011), which led us to hypothesize that MT modulates ADRA1d mRNA directly in these areas. We present evidence that supports a relationship between MT and NE in NCM and CMM. The NCM and CMM have specialized function for song recognition (Mello, Vicario et al. 1992; Mello and Clayton 1994; Bolhuis, Zijlstra et al. 2000) and thus nonapeptides may modify female song perception via interaction with the noradrenergic system.

However, OTA treatment did not significantly affect female behavior in the two-choice task. Previous study has shown that MT mRNA is elevated in the PVN of paired females (Lowrey & Tomaszycki, 2014) and treatment with an OTA increased latency to pair and decreased pair formation, especially in females (Pedersen & Tomaszycki, 2012). There is also evidence that destroying NE fibers alters females' ability to evaluate male song and decreases the likelihood to pair (Appeltants, Del Negro et al. 2002; Vahaba, Lacey, & Tomaszycki, 2013; Vyas, Harding et al. 2008). However, we did not assess these aspects of courtship behavior. The

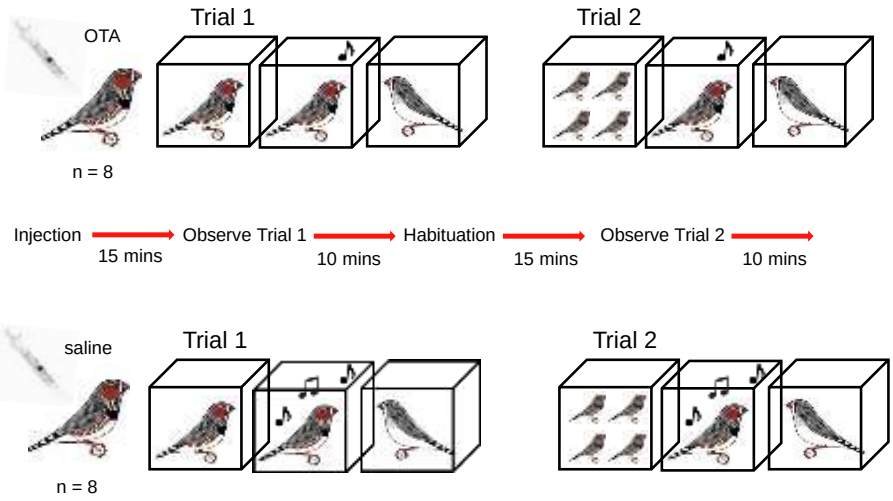
two-choice test only determines preference, so we cannot presently comment on possible differences in motivation or pairing behaviors in females. Therefore, it is likely that nonapeptides act upon the noradrenergic system in the NCM and CMM to alter female song perception that is reflected in pairing behavior, but not preference. This may be a result of the un-naturalistic nature of the two-choice paradigm.

Conclusion

This novel study is the first to investigate the relationship between MT and NE in the zebra finches. We found that treatment with an OTA lowers ADRA mRNA in both sexes. ADRA2c mRNA was lower in Area X and LMAN of treated males and ADRA1d mRNA was lower in the NCM and CMM of treated females. However, OTA administration did not affect preference within a two-choice paradigm. We discuss alternate mechanisms of action of the nonapeptide system on pairing via the noradrenergic system.

Figure 5.1. Model of the experimental design for males and females.

Males:



Females:

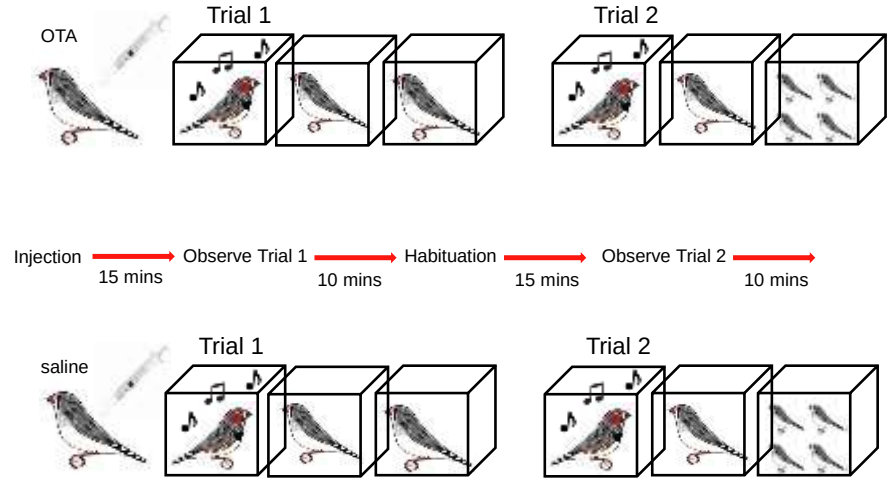
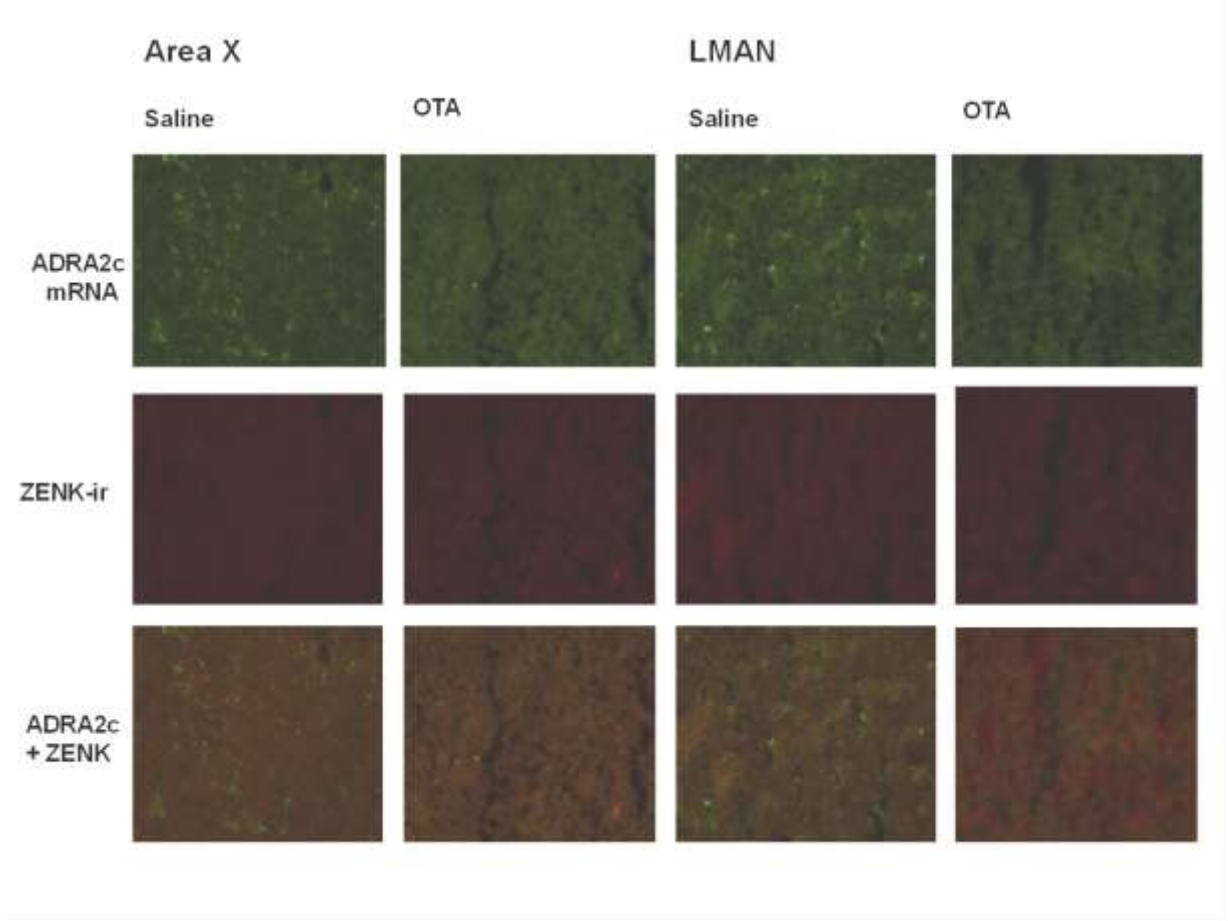
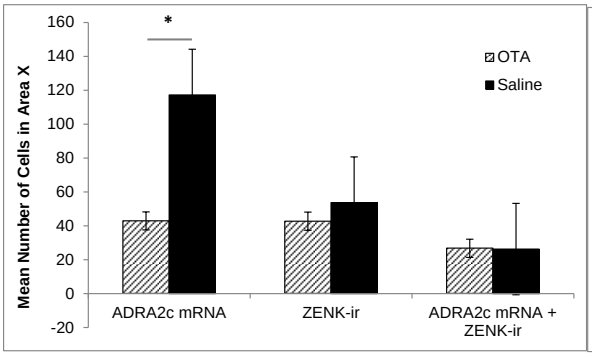


Figure 5.2. Examples of ADRA2c mRNA and ZENK-ir in Area X and LMAN of adult male zebra finches treated with OTA or saline. 400X magnification. Bar graphs represent the mean number of ADRA2c mRNA, ZENK-ir, and ADRA2c+ZENK cells for Area X and LMAN of adult male zebra finches treated with OTA or saline. Statistical significance is indicated by the * and error bars represent the standard error.





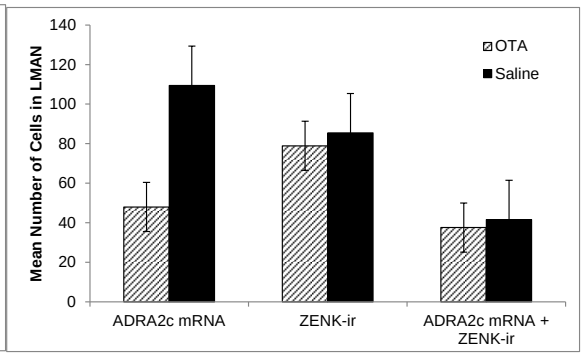
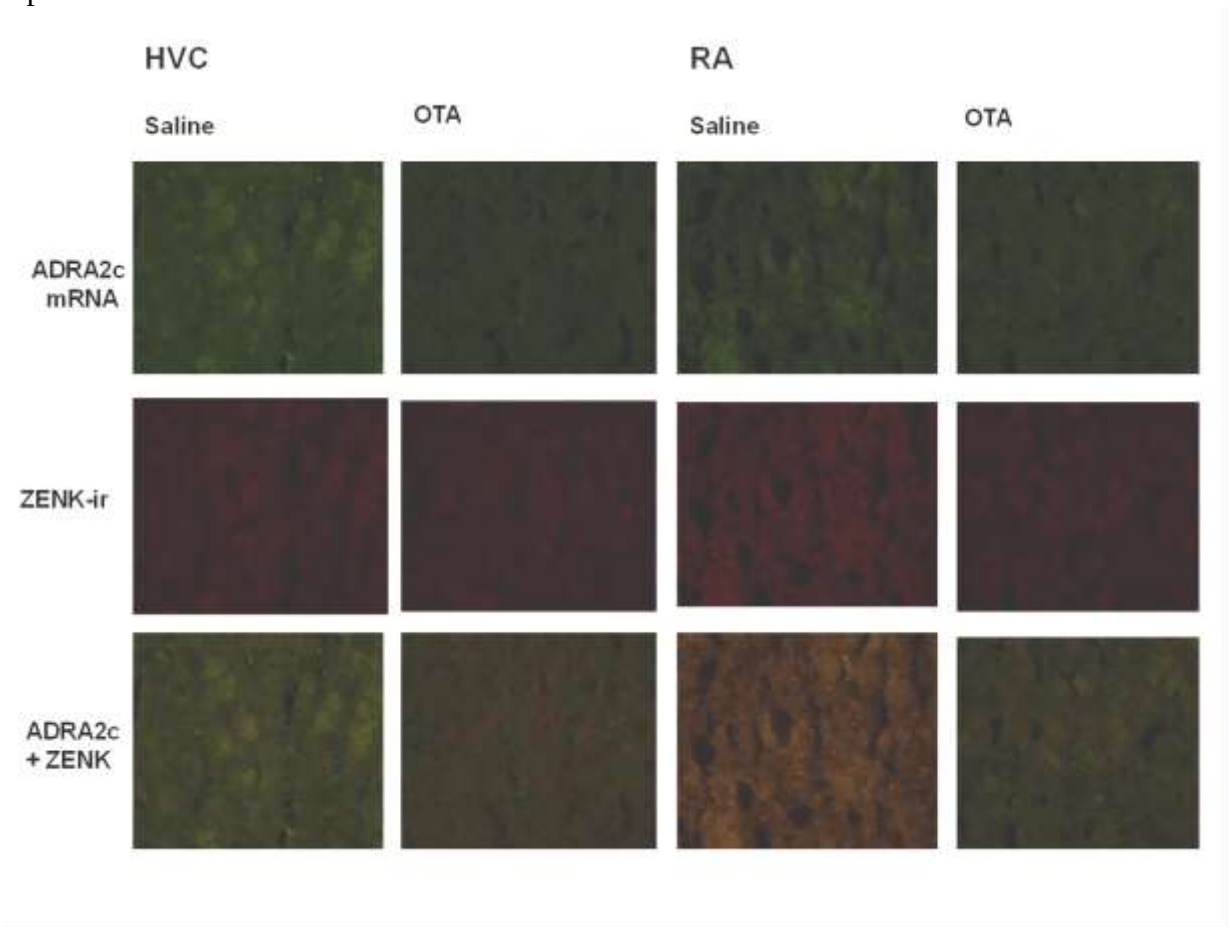


Figure 5.3. Examples of ADRA2c mRNA and ZENK-ir in HVC and RA of adult male zebra finches treated with OTA or saline. 400X magnification. Bar graphs represent the mean number of ADRA2c mRNA, ZENK-ir, and ADRA2c+ZENK cells for HVC and RA of adult male zebra finches treated with OTA or saline. Statistical significance is indicated by the * and error bars represent the standard error.



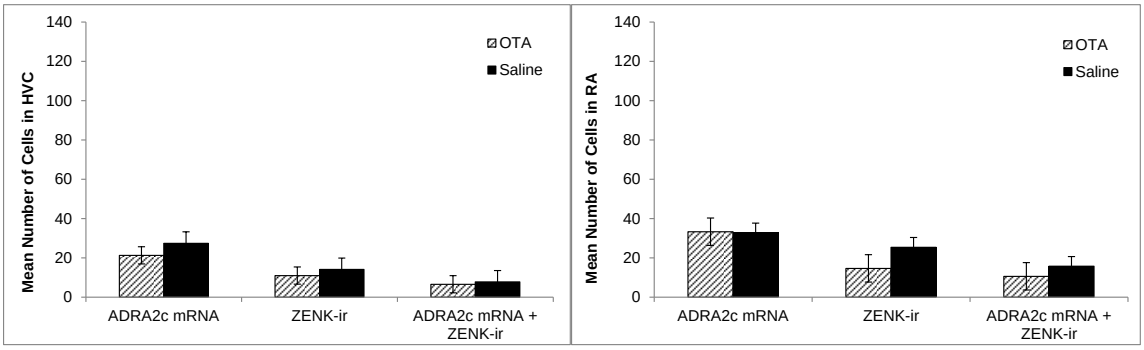
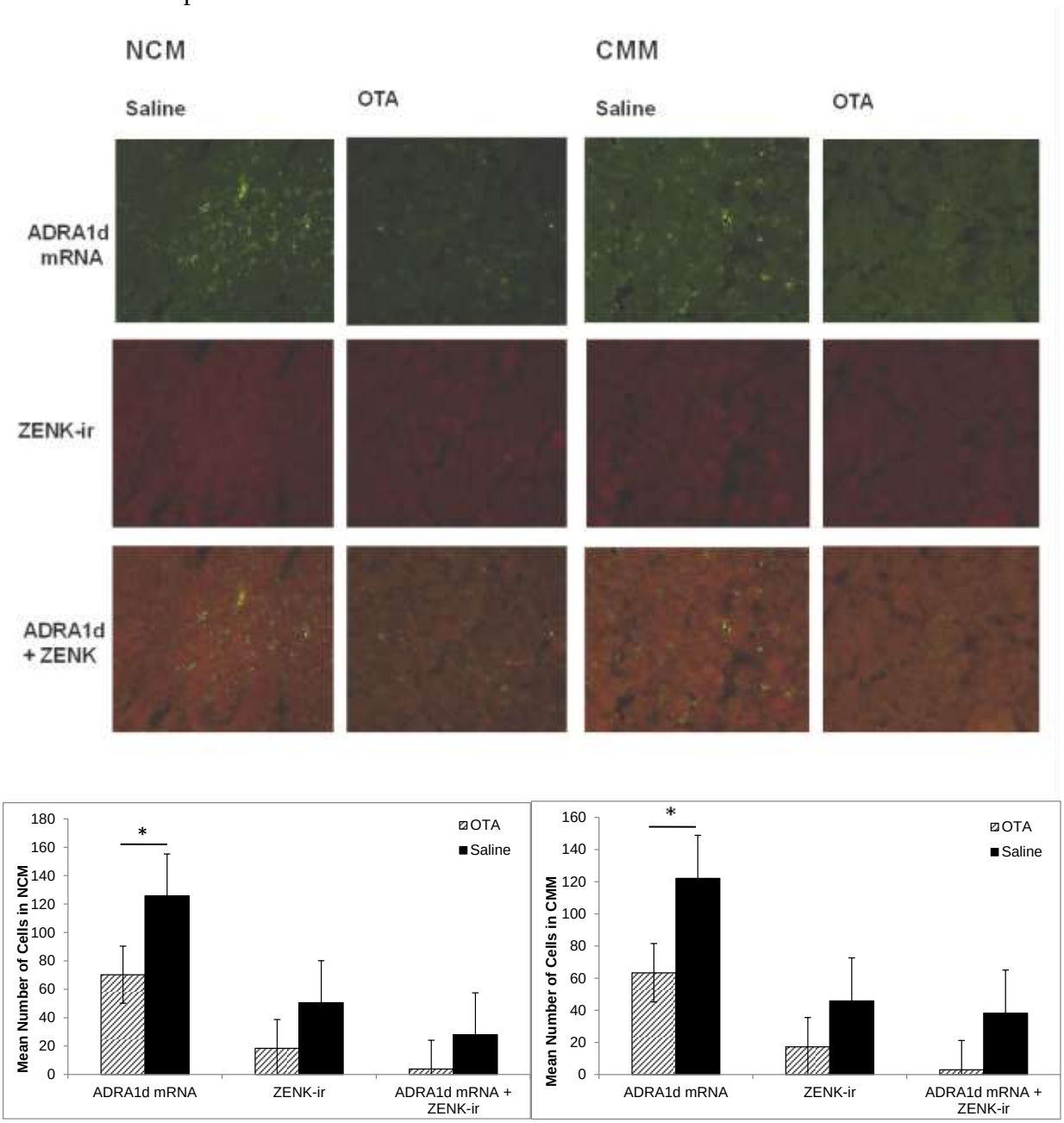


Figure 5.4. Examples of ADRA1d mRNA and ZENK-ir in NCM and CMM of adult female zebra finches treated with OTA or saline. 400X magnification. Bar graphs represent the mean number of ADRA1d mRNA, ZENK-ir, and ADRA1d+ZENK cells for NCM and CMM of adult female zebra finches treated with OTA or saline. Statistical significance is indicated by the * and error bars represent the standard error.



CHAPTER 6-SUMMARY OF FINDINGS AND GENERAL DISCUSSION

Hypothesis for Ch.2: Creating a social relationship, such as a monogamous pairbond, will increase nonapeptide expression in the PVN and BSTm of both male and female zebra finches.

The first experiment supported our original hypothesis and established that the nonapeptides MT and VT are involved in the formation of social relationship in both male and female zebra finches. Forming a monogamous pair bond resulted in higher MT mRNA and VT mRNA levels in the PVN of both male and female zebra finches. Increased MT and VT mRNA in the PVN of males was driven primarily by directed singing behavior, which plays a significant role in courtship. Increased MT mRNA in the PVN of females was driven primarily by clumping, which is a significant measure of female choice (Zann, 1996). These findings are important because the PVN is responsible for simultaneous secretion of nonapeptides into the bloodstream and the brain (Goodson, 2005; Neumann, 2002). The nonapeptides oxytocin (OT) and vasopressin (AVP) are widely implicated in social behavior (Donaldson & Young, 2008; Goodson & Thompson, 2010; Young & Wang, 2004). Therefore, MT and VT likely play a significant role in the formation of social relationships in both male and female zebra finches.

Hypothesis for Ch.3: Forming a social relationship, such as a monogamous pairbond, increases expression of catecholaminergic protein in the social behavior networks and auditory regions of both male and female zebra finches.

In males, we found increased TH+ZENK-ir in Area X of paired males versus unpaired males. This is consistent with previous findings and our hypothesis. However, none of the other song or social behavior areas was significantly different as a result of pairing. Also, none of the courtship or pairing behaviors were related to expression in Area X. In paired females, there

were decreased levels of TH+ZENK-ir in the VMH and increased levels in the VTAc. In the VTAc of paired females, TH+ZENK-ir was positively associated with nesting behavior and TH+ZENK-ir was positively associated with clumping. Nesting behavior involves both partners and is shared activity between bonded partners whereas clumping is a sign of female choice and initially occurs earlier in the pairing process (Zann, 1996), which would be considered a production of pairing behavior. It may be that female brain areas react differently throughout the formation of the relationship — such that activity in areas associated with social behavior, such as the VMH, may peak during courtship and areas involved in sexual reward, such as the VTA, may peak during the transition to a pairbond and preparation for parenthood.

We found some evidence to support our hypothesis, but the majority of the areas we expected to have increased TH+ZENK-ir in paired birds were not significant, such as song areas LMAN and HVC, reward area SN, and auditory area CMM. Further study would be needed to determine if dopamine is playing a significant role in the courtship and pairing process.

Hypothesis for Ch.4: Creating a social relationship, such as a monogamous pairbond, will increase activation of noradrenergic receptors in song and auditory regions of both male and female zebra finches.

The results of our previous study were not fully supportive of our hypothesis, so to fully understand catecholaminergic interactions with courtship and pairing behaviors, we decided to investigate the effect of pairing on noradrenergic receptor class ADRA. We found that paired males had increased levels of ADRA2c+ZENK-ir co-expression in Area X and LMAN compared to unpaired males. Paired females also showed increased levels of ADRA1d mRNA expression in NCM and CMM. Together, these findings do suggest that NE is active during the formation

of social relationships in both male and female zebra finches. Based on these findings, NE is likely involved in the formation of social relationships in zebra finches given the prevalence of NE in the brain - which contains 10 times the amount of NE compared to mammals (Barclay and Harding 1988; Waterman and Harding 2008).

In our previous study, TH-ZENK-ir co-expression was higher in Area X of paired than unpaired male zebra finches. The same birds were used in this study, but molecular markers were used for the noradrenergic receptor, ADRA2c, instead of TH-ir (which is a more general marker of catecholaminergic action). Therefore, all behavioral observations and levels of ZENK are consistent across these two studies. This means that the TH+ZENK-ir from Ch.3 could indicate noradrenergic action, as pairing had a significant effect on ADRA2c levels. It is also possible that both DA and NE are acting in concert in Area X during the courtship and pairing process, as Area X contains innervations of both fibers. However, due to the fact that ADRA2c+ZENK co-expression is higher in paired birds in both Area X and LMAN in the present study, it is likely that the TH+ZENK-ir increase seen in the same paired bird in our previous study was a result of NE action in Area X.

Hypothesis for Ch.5: OT modulates expression of noradrenergic mRNA expression in both male and female zebra finches and effects social choice in both sexes.

The final experiment in this series was designed to bring the previous findings together and establish a relationship between OT and NE in the formation of social relationship in the zebra finch. Blocking OT using an antagonist caused a decrease in ADRA2c mRNA levels in Area X of males and ADRA1d mRNA in the NCM and CMM of females. Given that the previous experiment showed that pairing increased mRNA levels in these same areas, this is

strong evidence the OT has a significant effect on NE during the formation of social relationships in the zebra finch.

Previous findings in rodents show that subchronic treatment with OT increases the responsiveness of ADRA2 to an alpha2 agonist in the PVN (Diaz-Cabiale et al. 2000), and the LoC (Petersson et al., 1998). Findings from avian neuroanatomy (Korf, 1984) demonstrate that the PVN (the site of OT release) directly innervates the VTA (a reward area), and LoC (the site of NE release). The LoC has direct noradrenergic innervations to the VTA (Mello, Pinaud, & Ribeiro, 1998) and Area X (Castelino, Diekamp, & Ball, 2007). Therefore, there are several possible routes for OT to affect NE. Therefore, I hypothesized that exogenous MT (originating in the PVN) is released in response to courtship behaviors, specifically directed song, and increases the level of ADRA2c mRNA in male zebra finches, which is the most prevalent receptor class within the song system (Velho et al., 2012). In female zebra finches, ADRA1d is the most prevalent receptor in the NCM and was found in more song-responsive cells in the NCM than other receptor types (Velho et al. 2012). MT receptors have also been found in the NCM and CMM (Leung et al. 2011). Therefore, I hypothesized that exogenous MT (originating in the PVN) is released in response to courtship behaviors and increases the level of ADRA1d mRNA in auditory regions of female zebra finches. My findings support these hypotheses.

Conclusions and Future Directions

The relationship between OT and NE systems in the formation of social relationships is complex and deserves further study to verify the results found in the present collection of studies. I believe that larger samples are needed in future research to confirm the findings presented here and to allow for more complex statistical testing. Other methods of measuring molecular activity

would also be useful for validating the present findings, such as HPLC, tract tracing, viral vectors, IEG, etc. My studies are part of a small group of research that has been done on social behavior in female animals. Little is known about female behavior, particularly in the zebra finch. Further study with female zebra finches can help us to design better experimental designs that elucidate the complex and subtle behavior of these fascinating social creatures.

The role of nonapeptides in social relationships has been well studied, but the role of NE is not understood. ADRA2c mRNA is localized to the basal ganglia, cerebral cortex and hippocampus (Scheinin et al., 1994), brain areas involved in memory processing. Activation of ADRA2c increases the synthesis of glycogen in the forebrain and inhibiting glycogen breakdown impairs memory formation (Gibbs, Hutchinson, & Hertz, 2008; Hutchinson, Gibbs, & Summers, 2008). ADRAs have been implicated in memory processing (Scheinin et al., 1994; Gibbs, Hutchinson, & Hertz., 2008; Hutchinson, Gibbs, & Summers, 2008; Gibbs, Hutchinson, & Summers, 2010) and in the song system (Riters & Ball, 2002; Cornil, Castelino, & Ball, 2008). Therefore, I hypothesize that NE and OT are interacting through ADRA receptors to affect social memory, which is necessary for the formation of social relationship.

In males, hypothesis for Ch.2 was supported by the findings that forming a pairbond was associated with increases in MT and VT mRNA in the PVN, the site of nonapeptide synthesis, and VT mRNA in the BSTm, a part of the social behavior network. This finding indicates that the nonapeptides are involved in the formation of the pairbond. TH+ZENK-ir was higher in Area X of paired than unpaired males, which partially supported hypothesis for Ch. 3 and a similar pattern was found in the expression of ADRA2c+ZENK-ir in Area X and LMAN, which supported hypothesis for Ch. 4. These findings suggest that NE is active in the song areas of

male zebra finches during the pairing process. It is possible that the TH+ZENK-ir activation seen in the second experiment was DA and not NE, as TH+ZENK-ir expression was not significantly different in the LMAN of paired and unpaired males. Blocking OT significantly decreased levels of ADRA2c mRNA in males, supporting hypothesis for Ch. 5 and indicating that there is a relationship between OT and NE in the zebra finch brain. This finding is similar to previous research in rodents, so these findings are the first to show such results in a socially monogamous species, such as the zebra finch.

Similar to males, paired female zebra finches also exhibited increased levels of MT and VT mRNA in the PVN, supporting hypothesis for Ch. 2 and indicating that nonapeptides play a role in pairbond formation. ZENK-ir levels were higher in the NCM and VMH of paired females, but lower in the VTA compared to unpaired females, indicating that the NCM and VMH are more active during the first 48 hours of pairbond formation. TH+ZENK-ir levels were higher in the VTAc of paired females, but lower in the VMH compared to unpaired females. This indicates that DA was more active in the VTAc of paired females during pairbond formation. Together, these findings partially supported hypothesis for Ch. 3 and indicate that areas of the female brain are selectively activated during the pairing process. Paired females exhibited higher levels of ADRA1d in the NCM and CMM than unpaired females, which supports hypothesis 4 and indicates that NE is effected by the pairing process. Given that only ZENK-ir was significant in the NCM of paired females in Ch. 3, it is likely that this was driven by NE activity rather than DA activity, as TH-ir and TH+ZENK-ir were not significant in the study. In the final study, blocking OT was related to decreased levels of ADRA1d in the NCM and CMM, supporting hypothesis for Ch. 5. These results demonstrate that both OT and NE are

involved in the formation of pairbonds in female zebra finches and that a relationship exists between OT and NE, involving ADRA1d receptors.

Better understanding the relationship between OT and NE activation in relation to social relationships has significant impacts on the study of psychological disorders. There are several disorders that have been linked to both OT and NE, such as depression, anxiety, schizophrenia, and autism (Castelino and Schmidt 2010; Cochran, Fallon, et al., 2013). One of these disorders in particular, autism, is characterized by abnormalities in speech and communication, impaired social functioning, and restricted interests (American Psychiatric Association, 2000). One study found that administering OT to male humans clinically diagnosed with autism facilitated the processing and retention of social information, specifically the comprehension of affective speech (Hollander, Bartz, et al., 2007). Previous animal research has shown that OT is related to social recognition (Dluzen, Muraoka, & Landgraf, 1998) and social memory (Griffin & Taylor, 1995) in the rat. Given that NE action at ADRA receptors is implicated in memory processing (Scheinin et al., 1994; Gibbs, Hutchinson, & Hertz., 2008; Hutchinson, Gibbs, & Summers, 2008; Gibbs, Hutchinson, & Summers, 2010) and that previous research and our current findings show that blocking OT has an effect on ADRA action, it is highly likely that NE and OT are interacting through ADRA receptors to affect social memory. This mechanism may be responsible for disturbances in social memory formation and even more broadly, social cognition, as seen in autism spectrum disorder. The results of the current group of studies are the first step toward understanding the role of NE and OT in the formation of social relationships. Further study is warranted, as there may be implications for psychological disorders, such as autism.

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ABSTRACT

THE EFFECTS OF COURTSHIP AND PAIRING BEHAVIOR ON THE NONAPEPTIDE AND NORADRENERGIC SYSTEMS OF ADULT MALE AND FEMALE ZEBRA FINCHES

by

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Social relationships are complex and likely involve the multiple neural circuits, including those involved in learning, memory, motivation, and attention. Two neurotransmitter pathways highly involved in these neural circuits are norepinephrine (NE) and the nonapeptides, vasopressin (AVP) and oxytocin (OT). There is extensive research implicating a role for the nonapeptides in trust, sociality, parental care, and romantic relationships. There is little direct evidence for the role of nonapeptides in monogamous relationships in any species other than the prairie vole (Goodson 2013). However, there is evidence that nonapeptides are important in pair bonding for both male and female zebra finches (Lowrey & Tomaszycki, 2014) and treatment with an OT antagonist results in an increased latency to pair in both sexes (Pederson & Tomaszycki, 2012). Thus, the role of nonapeptides in monogamous social behavior deserves further investigation. The role of NE in the avian song system has also been extensively studied,

but, to date, only one study has examined the role of NE in courtship. Depleting NE in male zebra finches resulted in a decrease in courtship behaviors and a latency to form a pair bond (Vahaba, Lacey, & Tomaszycki, 2013), indicating that NE does play a role in courtship and pair bonding. I hypothesize that OT modulates the noradrenergic system by increasing the level of alpha-adrenoceptor (ADRA) mRNA within the song system and auditory system to mediate male courtship behaviors and female choice.

AUTOBIOGRAPHICAL STATEMENT

I earned a B.B.A. in Business Marketing from James Madison University in 2003. I worked in the marketing field for 2 years in Washington D.C. before deciding that I wanted to pursue an advanced degree in a field I was more passionate about, which was psychology, and more specifically neuroscience. I earned a B.S. in Psychology with a concentration in Neuroscience from Pennsylvania State University in 2007. I began the Experimental Psychology M.A. program at American University in the fall of 2007. I was accepted into the Ph.D. program in Psychology, Behavioral and Cognitive Neuroscience at Wayne State University in 2009 as a Rumble fellow. After discussing my situation at American University with my advisor, Dr. Michelle Tomaszycki, I decided to transfer my graduate credits from American University to Wayne State University and complete my graduate studies there. In 2012 I completed my Master of Arts degree examining the effects of dopamine agonists and antagonists on courtship and pairing behaviors in male and female zebra finches. Following my masters I, decided to explore the interaction between the catecholamines and nonapeptides in the formation of social relationships in the zebra finch.